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A Histochemical Study of Plant Diseases Associated with Mycoplasmalike Organisms

by

Andrea Therese Anne da Rocha

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

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IN

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Date.....October 1, 1985.....

Abstract

A histochemical study was carried out on four mycoplasmalike organism (MLO) diseases; Eastern aster yellows (EAY, a New York isolate), Western aster yellows (AY27, an Alberta isolate), potato witches'-broom (PWB) and clover proliferation (CP). The host plant was periwinkle, Catharanthus roseus (formerly Vinca rosea). Four diagnostic techniques were investigated. The use of fluorochrome 4',6-diamidino-2-phenylindole.2HCl (DAPI) proved to be a rapid and simple technique for detecting MLOs. Thick sections from diseased plants stained with DAPI exhibited DAPI fluorescence in the phloem. Healthy controls had no DAPI fluorescence in the phloem. A modified indirect immunofluorescence (IF) method was developed for use with thick plant sections. Highly specific fluorescence was detected in the phloem elements of diseased tissues. The technique was rapid and simple and could be applied to the diagnosis of MLO diseases in plants. This is the first successful application of IF in plant mycoplasmology. Transmission electron microscopy (TEM) was the most reliable technique for the identification of MLOs. Scanning electron microscopy (SEM) appeared to be of limited use in detecting MLOs in plant tissues. Both electron microscope techniques were considered too lengthy, complex and costly for routine diagnostic testing.

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I. General Introduction

Mycoplasmalike organisms (MLOs) are associated with over 100 plant diseases. Characteristic symptoms of these diseases include stunted plants, yellow foliage, witches'-broom, excessive root development, virescent flowers, eventual cessation of blossoming and a tendency to break dormancy of axillary buds, corms, bulbs and tubers. MLOs infect an extremely large number of plants; "yellows" has been found in some 300 species of crop and weed plants in 48 families. In Alberta, MLO-associated plant diseases are important in several crops. MLO infections were of three symptom types: aster yellows (Chen and Hiruki, 1977, 1978b), potato witches'-broom (Wright, 1957) and clover proliferation (Chen and Hiruki, 1975, 1978a). MLO of aster yellows have caused sporadic to severe damage to economic plants such as small fruits, vegetables, ornamentals, grains and forage crops and to weed species (Hiruki and Chen, 1984).

Diseases caused by MLOs in plants have been recognized for many years - as early as 1666 clover phyllody was described (Daniels, 1979). However, the concept that the etiological agents are MLOs, organisms possibly related to animal mycoplasmas, is relatively new. Mycoplasma is the common name given to all members of the class Mollicutes. These organisms are wall-less prokaryotes which contain bacterial type ribosomes, RNA and DNA, bound by the plasma membrane. Mycoplasmatales, the only order in the class, is

subdivided into three families, Mycoplasmataceae, Acholeplasmataceae and Spiroplasmataceae. Spiroplasma, the only genus in the family Spiroplasmataceae, is morphologically distinguishable from other Mollicutes in being helical and highly motile. The other mycoplasmas are pleomorphic, having no rigid form. Mycoplasmas and acholeplasmas differ in their requirement for sterol, their genome size and the intracellular localisation of NADH oxidase. Two other wall-less prokaryote genera, Thermoplasma and Anaeroplasma, are of uncertain taxonomic position (Archer and Daniels, 1982).

Doi et al. (1967) first observed microorganisms in the phloem elements of plants infected with mulberry dwarf, potato witches'-broom, aster yellows and paulownia witches'-broom by transmission electron microscopy. On the basis of their ultrastructure, they were described as "mycoplasma- like". Due to the inability to culture the MLOs, they have as yet not been confirmed as true mycoplasmas and retain the name mycoplasmalike organism. In literature, however, mycoplasma has been used as a trivial name to describe the MLO.

MLOs are transmitted from the phloem of diseased plants into healthy plant phloem via the mouthparts of leafhoppers and other sap-sucking insects of the sub-order Auchenorrhyncha. Leafhoppers become viruliferous usually 9 to 16 days after feeding on infected plants. Viruliferous leafhoppers then inject the MLOs through the stylets into

the phloem cells of a new host plant. Translocation through the phloem is rapid because the sieve plate pores of the phloem cells do not act as effective barriers.

Diagnosis of diseased plants relies heavily on symptomatology, although similar symptoms have been known to occur in plants free of MLO infection. Electron microscopy is a sure method of MLO detection. This technique, however, is not of practical diagnostic value, due to the cost and complexity of the procedure. In search of a rapid and effective diagnostic method to detect MLOs in diseased plants, a histochemical study of plant diseases associated with MLOs was carried out. The main objectives were as follows:

1. to demonstrate the DAPI fluorescence in the phloem of plants infected with MLOs using the 4',6-diamidino-2-phenylindole.2HCl (DAPI) stain.
2. to compare the DAPI staining method with the immunofluorescence method of detection of plant MLOs.
3. to demonstrate the presence of MLOs in the phloem of the plants that give a positive reaction with DAPI by transmission and scanning electron microscopy.

II. Literature Review

A. Histochemical staining techniques

Electron microscopy, although the most reliable method of detecting MLO in plant tissue, is a complex and time-consuming technique. In their search for a faster, easier method, researchers have turned to light and fluorescence microscopy. These have been used, often in conjunction with histochemical stains, to develop diagnostic techniques.

Light microscopy studies of Ghosh et al. (1974) revealed Giemsa, lacmoid and Mann's stains to be satisfactory for the detection of MLO infection. The phloem of the diseased plant was differentially stained. Other stains used in light microscopy were gallocyanine-chromalum (Petzold and Marwitz, 1980a; Grunewaldt-Stöcker, 1982) which stained DNA and RNA and Dienes' stain (Deeley et al., 1979; Fletcher et al., 1984; Ghosh et al., 1984), which was known to stain animal mycoplasma (Dienes et al., 1948). Dienes' stain revealed plant MLOs as well.

With the fluorescence microscope, stains used were Aniline Blue (Hiruki and Dijkstra, 1973; Hiruki and Shukla, 1973; Dijkstra and Hiruki, 1974; Hiruki et al., 1974; Gocio and Dale, 1982; Ghosh et al., 1974, 1984) which stains callose in the phloem tissue, Fast Green and Azure I (Goszdziewski and Petzold, 1975) for MLO and host cytoplasm. Feulgen with auramine O as Schiff's reagent and

N,N'-diethylpseudoisocyanine chloride (Petzold and Marwitz, 1979a,b) for DNA and berberine sulphate (Petzold and Marwitz, 1979c; Grunewaldt-Stöcker, 1982) for DNA and RNA.

The use of light microscopy to detect disease specific fluorescence without the use of stains was also reported to be a useful tool for detecting . Researchers have used the fluorescence of phloem (Marwitz and Petzold, 1980; Namba et al., 1981), more specifically the fluorescence due to callose (Silvere, 1983a,b) and biogenic amines produced by aldehyde fixation and paraffin embedding (Petzold and Marwitz, 1984). Lee and Davis (1983) proposed the use of dark-field microscopy for the detection of MLOs. Isolation of the sieve tubes from the plant tissue was required for this.

The histological techniques described were all reported to have the ability to differentiate between healthy and MLO-diseased tissue. With the exception of Dienes' stain, they are nonspecific stains. Some detect the presence of nucleic acids in general. Others detect changes in the phloem tissue and thus indirectly indicate MLO infection.

B. DAPI staining

Introduction

There have been numerous reports of the use of a DNA specific stain, 4',6-diamidino-2-phenylindole.2HCl (DAPI), for the detection of MLOs in diseased plant tissue (Table

1). DAPI was first synthesized as a trypanocide (Dann et al., 1971). Williamson and Fennell (1975) reported that DAPI possessed useful DNA-binding properties. In yeast DNA studies, DAPI was shown to bind preferentially to adenine-thymine (AT) rich yeast mitochondrial DNA, forming a highly specific fluorescent complex.

In 1975, Russell et al. developed a simple cytochemical technique for detecting mycoplasma and virus contamination of tissue culture systems. This sensitive procedure involved adding DAPI to tissue culture cells on a coverslip at a concentration of 0.1 μg/ml in phosphate buffered saline (PBS) at 37°C for 15-30 min. The DAPI solution was then washed off with PBS and the coverslip examined in a fluorescence microscope. The fluorochrome was rapidly taken up by DNA yielding highly fluorescent nuclei and no cytoplasmic fluorescence. If the cells were contaminated with mycoplasmas, characteristic discrete fluorescent foci were readily detected in the cytoplasm and on the surfaces of the cells.

Application to plant MLOs

DAPI was used to demonstrate plant MLOs in the phloem of trees with pear decline or proliferation by Seemüller in 1976(a). Samples were fixed in 5% glutaraldehyde or 4% formalin in 0.1 M phosphate buffer, pH 7.0 at 4°C. After washing in the phosphate buffer, the samples were either fixed with osmium tetroxide for later examination or

Table 1 : The use of 4',6-diamidino-2-phenylindole.2HCl (DAPI) for the detection of mycoplasmalike organisms in diseased plant tissue

PLANT		DISEASE	REF.
Apple	Malus sylvestris Mill.	proliferation	1,2 3 4 5,6
Blackberry	Rubus sp.	Rubus stunt	3
Blueberry	Vaccinium corymbosum L.	blueberry stunt	7
China aster	Callistephus chinensis (L.) Nees	aster yellows	8
Chokecherry	Prunus virginiana L.	X-disease	9
Clover	Trifolium repens L.	phyllody	3 10
Coconut palm	Cocos nucifera L.	lethal disease	11 12 13
Dodder	Cuscuta subinclusa L.	phyllody spike stubborn	3 14 14
Hydrangea	Hydrangea macrophylla Ser.	virescence	10
Jujube	Zizyphus jujuba Mill.	witches'-broom	15
Mulberry	Morus sp.	mulberry dwarf	15
Orange	Citrus sinensis (L.) Osbeck	stubborn	10 14
Peach	Prunus persica L.	X-disease	9
Pear	Pyrus communis L.	decline	1,2 4 5,6

PLANT		DISEASE	REF.
Periwinkle	Catharanthus roseus (L.) Don	clover phyllody	10
			8
		Daucus proliferation	10
		virescence	10
		spike	14
		stubborn	14
		mulberry dwarf	15
		phyllody	3
		aster yellows	16,17
		potato witches' broom	16,17
		clover proliferation	16,17
Petunia	Petunia sp.	aster yellows	8
Sandal	Santalum album L.	spike	10
Speedwell	Veronica sp.	virescence	3
Wild tobacco	Nicotiana rustica L.	stobur	8

1 Seemüller, 1976a
2 Seemüller, 1976b
3 Cazelles, 1978
4 Schaper and Seemüller, 1982
5 Seemüller et al., 1984a
6 Seemüller et al., 1984b
7 Schaper and Converse, 1985
8 Petzold and Marwitz, 1980b
9 Douglas, 1984
10 Samyn and Welvaert, 1979
11 Schuiling et al., 1981
12 Nienhaus et al., 1982
13 Deutsch and Nienhaus, 1983
14 Hiruki, 1981
15 Bak and La, 1983
16 Hiruki and da Rocha, 1984a
17 Hiruki and da Rocha, 1984b

immediately cut into 20-30 μm thick sections with a cryomicrotome. The sections were stained with 1 μg/ml DAPI in phosphate buffer for 20-30 min at room temperature. After washing in buffer, the sections were viewed by fluorescence microscopy.

This procedure was followed in later studies with some modifications. Samples were often sectioned before fixing (Petzold and Marwitz, 1980b; Hiruki, 1981; Hiruki and da Rocha, 1984a,b) and in some cases, sections were made free-hand with a razor blade (Samyn and Welvaert, 1979; Hiruki, 1981; Schuiling et al., 1981; Nienhaus et al., 1982; Deutsch and Nienhaus, 1983; Hiruki and da Rocha, 1984). Schuiling and co-workers (1981), Nienhaus et al. (1982) and Deutsch and Nienhaus (1983) fixed the tissue at 4°C in 0.05 M sodium cacodylate buffer, pH 7, 0.15 M sucrose and 2 mM calcium chloride to which glutaraldehyde and paraformaldehyde were added to final concentrations of 3% each. In all other reports, 5% glutaraldehyde in 0.1 M phosphate buffer, pH 7 was used, usually for 2 hours. Petzold and Marwitz (1980b) embedded their sections in paraffin after fixing and stained the sections with DAPI at pH 7.2. Schuiling et al. (1981) used 0.1 μg/ml DAPI in 0.1 M phosphate buffer, pH 7 at room temperature for 5-15 min. Schaper and Converse (1985) used DAPI in 0.1 M phosphate buffer, pH 7.0 and mounted the sections in DAPI solution. Hiruki and da Rocha (1984a,b) used DAPI in an aqueous solution.

Results of DAPI staining of plant tissue were consistent in all reports. In healthy specimens, DAPI fluorescence was absent in the phloem region. Some fluorescence of mitochondrial DNA was observed, but this was weak. Typical autofluorescence of the xylem was observed in both healthy and diseased specimens. In MLO-infected specimens, a very clear and strong DAPI fluorescence in the sieve tubes was noticed (Schaper and Converse, 1985). Seemüller (1976b) described the fluorescence as small particles , mostly irregularly distributed, with many in one sieve tube and only a few or none in others. They were often concentrated in the sieve plate areas of the sieve tubes. Fluorescence observed was different from the fluorescence phenomena reported by other authors to occur in diseased phloem due to cell wall reactions or callose accumulations at the sieve areas. In comparison of the fluorescence to that described by Russell et al. (1975), the correspondence of the observations, the size of the particles and the fact that the particles occur in the sieve tubes led Seemüller (1976b) to believe that the fluorescence was related to the occurrence of MLOs.

Samyn and Welvaert (1979) studied both artificially-infected and naturally-infected plants. They reported a weaker DAPI fluorescence in naturally-infected plants than in the artifically-infected Catharanthus roseus. In the naturally-infected plants the fluorescence was also limited to a few spots. Considering the total absence of

fluorescence in healthy plants, it was felt that even a few fluorescent spots could be regarded as positive. DAPI fluorescence was also noticed in sections from plants without symptoms but which were known to be infected because of the presence of earlier symptoms or because of the observation of MLOs by electron microscopy. Plants that had witches'-broom-like symptoms but in which no MLOs were ever observed by electron microscopy, gave negative results with DAPI.

Hiruki (1981) reported that plants graft-inoculated with MLO for three weeks showed a moderate amount of MLO-specific DAPI fluorescence. It appeared that the amount and intensity of MLO-specific DAPI fluorescence was related to the severity of external symptoms. When aster yellows MLO-infected plants were examined 3 and 7 weeks after grafting, the latter showed more widely distributed fluorescence with high intensity. When a mild MLO disease and a severe MLO disease, both of which were rather consistent in symptomatology, were compared for MLO-specific fluorescence, the latter developed strong fluorescence sooner after grafting than did the former.

In a study on the condition of the phloem and the persistence of MLOs associated with apple proliferation and pear decline, Schaper and Seemüller (1982) observed a change in persistence and pattern of distribution of MLOs over winter within infected trees using DAPI staining. In the fall until the end of December, the MLOs were detectable as

single fluorescent particles. Where their numbers were sufficiently high, part or all of the sieve tube fluoresced. The MLO fluorescence was also present in nonfunctional sieve tubes; thus it seemed either that the MLOs remained alive after the tubes had degenerated or that they continued to fluoresce after death. During January and February, the MLOs in stem sections viewed by fluorescence microscopy appeared to be aggregated into "stringlike" structures. The "strings" were always located along the cell walls of the sieve tubes. In March and April, few typical DAPI reactions could be found and in many cases the "stringlike" structures had also disappeared. The degeneration of MLOs was more pronounced in orchard pear trees than in apples. In root systems, no major changes were noted in the persistence of MLOs as winter progressed. MLOs were always present in relatively high numbers. The tissue in which typical fluorescence patterns occurred were those in which the phloem was still functional. "Stringlike" structures were occasionally observed in degenerated sieve tubes in older parts of the phloem.

Quantitative results of DAPI staining have also been presented. DAPI staining gave positive results 0 out of 18 times with healthy tissue and 14 out of 16 times with tissue from pear decline and proliferation diseased plants (Seemüller, 1976a) . Schaper and Converse (1985) evaluated healthy and stunt-infected blueberry plants. DAPI was 96% correct in evaluating the diseased plants and 100% correct

in evaluating the healthy plants.

The efficiency of DAPI staining for the detection of MLO infection in plants has been compared to the use of other histochemical stains. In all tests, DAPI proved to be equal or superior. In the initial work on the use of DAPI in phytopathology, Seemüller (1976a) compared it to aniline blue. Test results showed DAPI to give correct results 14 out of 16 times. Aniline blue gave correct results 12 out of 16 times. In healthy control tests, both stains were correct 18 out of 18 times. In a later report, Seemüller (1976b) stated that both stains gave essentially the same results, and therefore DAPI might possibly offer an opportunity for the diagnosis of diseases caused by MLOs.

Samyn and Welvaert (1979) made successive cuttings of the same stems and treated them with aniline blue, fast green or DAPI. The difference between a diseased plant and a healthy one could be observed distinctly with all products. With the aniline blue, however, fluorescence could be observed even in healthy plants. With light or recent infections, results obtained from the cuttings treated with DAPI were more conclusive. The fluorescent spots were much more perceptible and permitted a more accurate diagnosis.

Hiruki (1981) considered both aniline blue and DAPI to be useful for the detection of MLO infected materials. He cautioned, however, that the presence of callose in healthy tissue demanded careful preparation of section samples and

cautious interpretation of results with aniline blue. With DAPI, staining of host DNA occurred in some species and in extreme cases this might interfere with the MLO test.

In investigations of the lethal disease of coconut palm, Nienhaus and co-workers (1982) found a close correlation between DNA accumulation detected by DAPI and callose formation in sieve cells detected by aniline blue. The latter, though, was considered to be nonspecific and due to early senescence of tissue. It indicated that the phloem had already reacted to the pathogen when it still looked unaffected macroscopically.

Bak and La (1983) investigated the efficiency of DAPI, aniline blue and quinacrine in the diagnosis of MLO infections in jujube, mulberry and periwinkle plants and reported that all were efficient techniques. DAPI appeared to be the most efficient.

Results from Schaper and Converse (1985) showed DAPI to be 96% correct with known stunt-infected blueberry root samples and aniline blue to be 76% correct with petiole samples. All 25 healthy root samples were scored correctly with DAPI, whereas 40% of the healthy petiole samples were scored correctly with aniline blue. Use of aniline blue to classify infected and healthy blueberry root sieve tubes instead of those in petioles did not improve the accuracy of their classification. Because callose deposits tended to increase as healthy sieve tubes aged, the aniline blue technique would probably be most reliable when used to

examine young sieve tubes. In addition, rapid fixation of tissues before aniline blue treatment might help to reduce formation of wound callose in tissue.

DAPI was also compared with a benzimidazole derivative Hoechst 33258 (Seemüller, 1976a,b). With this compound it was also possible to demonstrate the fluorescent MLO particles. However, it seemed to be less suitable because it caused much more background fluorescence in the plant tissue than DAPI. It also caused fluorescence of callose that might complicate the identification of the particles.

Histochemical stains used with light microscopy have been also compared with the DAPI staining technique. Sections treated with Dienes' stain were compared to DAPI-treated sections of lethal diseased coconut palms (Deutsch and Nienhaus, 1983). The same cells in the phloem area reacted similarly. Although the Dienes' staining technique produced adequate results on DNA accumulation, the DAPI method seemed to be more sensitive.

With Schiff's Feulgen stain, good results were obtained (Cazelles, 1978). The nuclei were clearly visible and the sieve tubes were well stained and formed continuous passages interrupted only at the sieve plates. This staining was easy to detect in clover, Catharanthus roseus and Cuscuta subinclusa with phyllody or in Veronica with virescence. However, it was difficult to discern the cells at the start of colonization by the MLOs. The staining reaction was too weak. Identical results were obtained with DAPI, but the

details were more numerous. The sieve tubes were not only coloured en masse; but one could notice that this fluorescence was due to the presence of a large number of juxtaposed fluorescent-blue points.

In apple with proliferation or blackberry with Rubus stunt, the Feulgen type reaction was never positive except for a weak staining of the nuclei in the meristem region. With the DAPI stain, the concentrations of MLOs observed corresponded to the observations by electron microscopy. Consistent accumulation of fluorescent grains was found the sieve tubes of the apple tree. In blackberry tests, concentrations stronger than for the apple tree were observed, but weaker than those observed in Catharanthus or clover. Thus, Cazelles (1978) concluded that the Feulgen stain was satisfactory for use with certain herbaceous plants but it was not satisfactory for use with lignified plants such as apple, pear and blackberry.

Petzold and Marwitz (1980b) used DAPI in conjunction with berberine sulphate to detect MLOs in plant tissue. The different secondary fluorescence resulting from the application of both stains permitted an improved differentiation of the tissue and also the estimation of the amount of contamination by MLOs. DAPI stained only DNA and had a stronger binding power than berberine sulphate. Berberine sulphate, however, also stained RNA. The fluorescence produced by the two fluorochromes was almost complementary. DAPI gave a blue fluorescence and berberine

sulphate a yellowish fluorescence. Both stains possessed, moreover, a different affinity for the other cell components. The cell wall, especially that of the xylem, showed a higher affinity for berberine sulphate, so that DAPI was repressed and a bright yellow-green fluorescence was produced. The yellow-green fluorescence of the cytoplasm and plastids was due to the high portions of RNA. The colour and intensity of the sieve cells depended on the frequency of MLOs. A blue fluorescence pointed to a smaller number of MLOs. The DNA contained in the MLOs was bound by the stronger DAPI stain. When the amount of MLO increased, berberine sulphate stain affected the colour of the fluorescence. The thicker layer of MLOs had a higher concentration of RNA to which the berberine sulphate was bound. Thus the fluorescence produced was white-blue to white-yellow.

From the numerous reports on the use of DAPI, the consensus appeared to be that it was an effective stain for the detection of MLOs in plant tissues. Nienhaus et al. (1982) suggested that the DAPI stain, as a pretest, could provide a quick survey of tissue, as sections could readily be made with a razor blade. It facilitated the cumbersome and quantitatively-restrictive work with the electron microscope, which was necessary for the demonstration of phloem inhabiting, non-cultivative microorganisms. Samyn and Welvaert (1979) listed as an additional advantage of the stain that cuttings already treated with DAPI could be used

for further examination with the electron microscope.

Hiruki and da Rocha (1984b) described the technique as simple and practical, being suitable for assaying a large number of disease samples with high consistency. Seemüller et al. (1984a) expressed the opinion that the reliability of efforts to demonstrate MLO with the fluorescence technique in slightly or unevenly colonized trees depended on the number of samples examined. The same applied to materials from graft transmission experiments. Negative results were not conclusive when only one or two samples were examined or grafted.

Schaper and Converse (1985) discussed the DAPI technique in some detail. It was found reliable for distinguishing between blueberry stunt-infected and healthy blueberry plants. Blueberry roots were the most suitable organ to examine for MLOs by the DAPI technique. If a given root was colonized, usually a high MLO population could be detected in its sieve tubes. For symptomless plants, it was possible to fail to detect the blueberry stunt agent by only examining stem samples. If the blueberry cultivars had distinctive stunt symptoms, it apparently did not make much difference if root or stem samples were examined for MLO colonization. Therefore, for checking symptomless blueberry plants, examining root samples was recommended to reduce the amount of possible false negative evaluations.

The distinctive fluorescence pattern DAPI produced enabled MLO colonization in a sample to be rated from none

to light to heavy. In a laboratory where this test was performed routinely, an experienced worker could probably cut, stain and evaluate 30-40 tissue samples in a normal working day. Stunt-diseased blueberry plants were incorrectly classified in 4% of the root samples tested with the DAPI technique, whereas no errors were made in the classification of known healthy blueberry plants. With the DAPI technique for MLO detection, experience helped reduce errors in distinguishing between the very similar DAPI fluorescence that occurred with MLOs and with plant nuclei. Fluorescing nuclei can be a problem, especially in plants that are slightly diseased and are colonized only in the youngest sieve tubes. DAPI fluorescence due to nuclear DNA in such sieve tubes could be mistaken for fluorescence of MLOs. Mitochondria also could be mistaken for MLOs, but this is a minor problem. In contrast to MLO DNA, which was always intensively stained with DAPI, plant mitochondrial DNA was only occasionally apparent in stained preparations. In such cases, the DAPI fluorescence of mitochondria was weaker than that of MLOs. The mitochondrial DNA DAPI fluorescence was also quickly quenched when exposed to UV light, whereas the MLO DAPI fluorescence remained stable for at least several minutes under UV exposure.

Molecular mode of action

Williamson and Fennell (1975) were the first to use the DAPI compound as a DNA-binding fluorochrome. In aid of

their separation, an organic compound was sought that would bind differentially to the nuclear and mitochondrial DNA of yeast. The rationale of this approach was that DNA complexed with organic compounds almost always underwent a decrease in buoyant density in cesium chloride. Mitochondrial DNA had a molar AT content of about 83%, compared with only 61% for the main nuclear component and this made the mitochondrial DNA about 16 mg/cm^3 less dense than nuclear DNA in isopycnic cesium chloride gradients. Thus banding in the presence of a compound with a preference for AT base pairs should enhance the separation between the DNAs.

DAPI is related to the trypanocidal drug berenil, which was known to bind preferentially to AT-rich DNA. Berenil slightly enhanced the separation of yeast nuclear DNA and mitochondrial DNA in cesium chloride gradients, but not to a useful extent. DAPI, however, was found to give a striking separation of the two components and in addition, the complex it formed with DNA was highly fluorescent. The latter property not only added enormously to the value of the agent in the context of separating and detecting DNA in gradients, but also opened up an entirely different field of application, since DAPI could be used as a highly sensitive and specific fluorescent probe for DNA at the level of the individual cell. The excitation frequency of DAPI was about 365 nm and the main emission occurred at about 450 nm. The free dye was about as fluorescent as the dye-DNA complex.

The dye did not stain polysaccharide and probably did not stain RNA.

Kania and Fanning (1976) suggested that DAPI recognized the DNA sequence at the intercalation site, which was probably AT-rich. That same year, Schweizer (1976) showed that DAPI bound to the intercalary and terminal heterochromatic segments of chromosomes. A study on the molecular mode of action of DAPI using spectroscopic methods, thermodynamic procedures and the technique of affinity chromatography (Chandra and Mildner, 1979) produced the following results:

1. DAPI interacted with DNA and polydeoxynucleotides in a specific manner;
2. the complex formation between DAPI and DNA was greatly influenced by certain bivalent cations;
3. DAPI interacted preferentially to dAT-rich regions of the polymers. The drug exhibited a very high affinity towards the hybrid polymer, poly(rA).poly(dT);
4. at the molar ratio between DAPI and DNA of 0.05 to 0.15, the drug intercalates to DNA;
5. experiments to elucidate the nature of the bonds involved in the binding of drug to DNA indicated the involvement of hydrogen bonds, or other type of interactions.

Masotti*et al*. (1982) found that cations shown to influence DAPI-DNA binding were fluorescence quenchers. Evidence was presented that strongly supported the model where DAPI

intercalates into DNA. Zakarashvili et al. (1983) investigated the physiochemical properties of the DNA of two strains of mulberry dwarf MLO. The DNA of these strains had G+C contents of 34.2 and 34.5 mol%. Thus these strains would be AT-rich.

C. Immunological methods

Introduction

The use of immunological methods for detecting MLO antigens in plant extracts has been explored by several researchers. In all cases, the purification of MLO as antigen and its use in the preparation of antiserum was necessary. Sinha (1974, 1983a) developed a procedure for the purification of MLO cells from infected plants. It involved the following steps: i) infiltration of infected leaves with a magnesium chloride and glycine buffer under vacuum, ii) extraction of the sap, treatment with charcoal powder and absorption of the mycoplasma from the mixture by passing through a Celite pad, iii) high speed centrifugation of the eluted opalescent fractions, iv) fractionation of the concentrate through a Sepharose column, v) sucrose density gradient centrifugation and vi) high speed centrifugation to obtain a pellet of the purified mycoplasma. Over 500 batches of MLO infected leaves were subjected to this procedure with reproducible results. Recovery of the MLOs was low, varying from 0.7 to 1.7 g per

50 g of leaves. Approximately 75% of the mycoplasma was lost during the purification procedure.

Clark et al. (1983) introduced a procedure for the preparation of the "membrane fraction" (MF) from MLO infected Catharanthus roseus. This procedure involved extraction of sap in glycine buffer with magnesium chloride using a mortar and pestle. The sap was clarified by centrifugation and the supernatant subjected to high speed differential centrifugation. The resulting pellet was resuspended in buffer, then re-clarified by passing through a Millipore pre-filter and differentially centrifuged. This procedure resulted in pellets with considerable quantities of plant material. Thus a later cross-absorption of the antiserum produced with healthy plant material was necessary.

Other immunogens were prepared from insect vectors of MLOs. Caudwell et al. (1974, 1982) obtained extracts from the cicada (Euscelidius variegatus) carrying MLO of "flavescence dorée" of grapes. The extract was centrifuged at low speed and filtered through a 450 nm poresize filter to eliminate bacteria. It was then centrifuged at high speed to sediment the infectious particles. Lin and Chen (1985) prepared MLO immunogen from heads or salivary glands from approximately 2000 inoculative leafhoppers (Macrosteles fascifrons). Prior to the isolation of the MLO immunogen the leafhoppers were allowed to feed on aster yellows-infected lettuce for 3 days and subsequently

maintained on rye plants for 2 weeks to permit completion of the latent period. The dissected salivary glands were homogenized in 0.02 M phosphate buffered saline (PBS) and cleared by low speed centrifugation (2000g). The resulting supernatant liquid containing MLO was used as immunogen.

Antiserum was produced in most cases in rabbits. Sinha's inoculation schedule involved twelve weekly intramuscular injections of purified MLOs with adjuvant. Three weeks after the last injection, 2 additional weekly injections without adjuvant were given intravenously. One week after the last injection, the rabbit was bled. Clark et al. (1983) injected their rabbit at 2 to 3-week intervals with the partially purified membrane fraction and adjuvant at multiple sites (sub-cutaneous, intra-dermal and intra-muscular). Animals were bled at 7- to 10-day intervals beginning 6 to 8 weeks after the primary injection. Caudwell et al. (1974, 1982) used the purified extract from 300 cicadas for each injection. Three intravenous injections were given at 3-day intervals followed by an intra-muscular injection with adjuvant 10 days later.

Lin and Chen (1985) immunized 6-week old female BALB/c mice to produce monoclonal antibodies. Intra-peritoneal injections were given on days 1 and 28 and an intravenous injection on day 35, with 200 μg of protein per injection. The mice were killed on day 38. Murine myeloma cells were fused with splenic cells from the immunized mice and

hybridomas were selected. Screening was performed by indirect enzyme-linked immunosorbent assay (ELISA).

Several methods of serological detection of MLOs have been attempted. Sinha's laboratory at Agriculture Canada, Ottawa, used immunodiffusion (Sinha 1979, 1983b), ELISA and immunosorbent electron microscopy (ISEM) (Sinha and Benhamou, 1983; Sinha and Chiykowski, 1984). Caudwell (1982) also used ISEM to detect MLO causing "flavescence dorée" of grapes. Immunodiffusion tests of aster yellows antigen and antiserum showed two bands formed between the antigen and antiserum wells. Four days were required for the bands to appear with intact MLO cells but only 2 days with cytoplasmic antigens. The suspension of membranes did not produce any bands unless treated with detergent. The minimum concentration of MLO detectable was 20 mg/ml. When partially purified preparations were used, results were similar. Use of partially purified antigen greatly reduced the time required and the preparations contained about 90% more MLOs than the purified preparations. The immunodiffusion test was used to determine the relative concentration of MLOs in plants at various times after inoculation. ELISA tests gave positive reactions with MLO preparations of concentrations as low as 4 µg/ml. The minimum concentration required to trap the MLOs on the antiserum-coated grids in ISEM tests was about 1.25 µg/ml. Storage of the antiserum at -20°C for up to 4 months did not reduce its effectiveness. The specificity of the

serological reactions was tested. The MLOs found associated with clover phyllody and aster yellows could not be differentiated, whereas Spiroplasma citri, peach X and clover yellow edge could be serologically distinguished from aster yellows.

The ELISA technique was used by other researchers to examine the relatedness of various MLOs as well as their relation to spiroplasmas. No specific relationship was found between MLOs tested or between spiroplasmas and numerous MLOs (Clark et al., 1983; Townsend, 1983; deLeeuw et al., 1983; Lin and Chen, 1985).

Malinovskaya and Skripal (1983, 1984) used the passive hemagglutination reaction and the complement fixation test to study the antigenic interrelationships of plant MLOs. Their results suggested some relatedness between the MLOs, as well as their closer relatedness to acholeplasmas than to true mycoplasmas.

Immunofluorescence (IF)

The immunofluorescence (IF) test is a serological method that has been used in animal mycoplasmology (Barile and Grabowski, 1983; Gardella et al., 1983), plant spiroplasmology (Williamson, 1982) and especially in virology. It is an advanced technique of histochemistry, which provides a means of observing an antigen-antibody reaction by chemically linking a fluorescent dye, such as fluorescein isothiocyanate (FITC) to the antibody molecules.

Such labeled antibodies retain the ability to react specifically with their respective antigens and when viewed in a fluorescence microscope, the reaction site is usually detected by its fluorescence. Maximum absorption by FITC-IgG occurs at approximately 495 nm and emission occurs at 525 nm. Using a fluorescein-labeled antibody, Coons et al. (1942) succeeded for the first time in tracing soluble pneumococcal polysaccharide antigen in tissue sections of mice infected with pneumococcus.

The specificity of antigen-antibody reactions, coupled with the relatively short time required to prepare and observe the specimen, makes the IF procedure an ideal diagnostic tool. Well over 500 English-language references to IF methods in diagnostic virology alone could be listed. Two excellent reviews by Emmons and Riggs (1977) and Kawamura(1977) are recommended.

One method used in IF is the indirect method. First, a known antibody (primary antibody) is reacted with an unknown antigen. In the second step, the serum globulin of the antigen-antibody complex formed is stained with specific antiglobulin (secondary antibody) conjugated with a fluorochrome. If specific fluorescence is detected, we have indirect evidence of the presence of the specific antigen-antibody complex formed in the first step.

The merit of this method is that it is more sensitive than the direct method by 5 to 10 times. Maximum amounts of primary antibody react with this excess, yielding stronger

fluorescence than in the direct method. Another merit of this procedure lies in the small amount of primary antibody required in the test. A third advantage is that a conjugated secondary antibody such as antirabbit IgG FITC can be used with any primary antibody obtained from rabbit. This eliminates the necessity of conjugating each specific antibody with FITC. Also, several secondary antibody conjugates are commercially available.

D. Electron microscopy

Transmission electron microscopy (TEM) has played a major role in the detection of mycoplasmalike organisms (MLOs) in plant tissue ever since it was first used in the discovery of MLOs as a presumptive pathogenic agent in plant tissue by Doi et al. (1967). Several review articles on the technique have been published (Waters, 1982; Cole, 1983; Norris and McCoy, 1983). Because MLOs are small, readily deformable and often without easily defined shape, they require the resolution of electron microscopy. Not all features can be optimally resolved by use of any one preparative technique, but the two most basic techniques are negative staining and thin sectioning. Negative staining of whole specimens reveals principally surface features, but thin sections permit verification of single membrane enclosure and of prokaryotic internal structure.

The chemicals and techniques for TEM of MLOs are similar to those used for TEM sections of healthy phloem

tissue, since MLOs are found in the phloem sieve elements of plants. Any plant part containing phloem tissues may contain MLOs. In some plants, MLOs are relatively evenly distributed throughout the entire plant and are easily located at any phloem site. In other plants, however, MLO populations are highly localized, making tissue sectioning for MLO visualization critical. If the sampling site is not critical, young succulent tissues should be chosen, as they are easier to fix and embed.

Norris and McCoy (1983) suggest that if MLOs have not previously been reported in a particular species, collections should be made from several different sites on a plant. As not every witches'-broom or foliar yellowing is associated with MLOs, eliminating other possible causes of abnormalities such as mites, fungi or herbicide damage before TEM examination would save time and effort.

MLOs are recognized by their typical size, irregular rounded to filamentous shapes and the absence of a cell wall. The membrane, ribosomes and DNA strands are visible at higher magnifications. Cole (1983) describes a method to identify MLOs by TEM. Adequate magnification and a proper plane of section are required to resolve the "triple track" (dark-light-dark) profile of the 7 to 8 nm (width) plasma membrane and to affirm that no additional outer membrane or no homogeneous cell wall is present. Internally, the MLO cell contains ribosomes or polysomes, among which are interspersed lucent and irregular areas containing fine

strands that represent the nucleoid. Sometimes, depending on osmolality at the time of fixation, the nucleoids may be condensed and more or less spherical. Waters and Hunt (1980) revealed the morphology of MLOs in vivo by graphic reconstruction from ribbons of serial ultrathin sections containing 45 sections. The MLOs exhibited a range of different morphologies, even within the same sieve element.

MLOs are often found in immature to recently matured phloem, which may also contain vesicles and degenerating mitochondria that superficially resemble MLOs. Vesicles in young sieve elements or parenchyma may contain ribosomes and fibrous material. Esau et al. (1976) distinguished the MLOs by their size, by the absence of DNA strands in the vesicles and by the size and location of the ribosomes. Ribosomes of plant host origin are larger than MLO ribosomes and line the inner face of vesicles formed from rounded-up rough endoplasmic reticulum. Remnants of the double membrane and cisternae distinguish degenerating mitochondria; vesicles smaller than 0.1 μm diameter are too small to be MLOs.

Scanning electron microscopy (SEM) is a powerful tool for high resolution morphological studies of the MLOs.Articles on the use of SEM for studying mycoplasma and MLOs include those by Klainer and Pollack (1973), Waters, (1982) and Carson and Collier (1983). SEM provides the ability to view cell surfaces of mycoplasmas as well as their interaction with surfaces of host cells. However, the application of SEM to the study of MLOs has barely been

explored. Klainer and Pollack (1973) had attributed this to the false impression that the available resolving power would not allow adequate visualization of organisms other than larger microorganisms. Yamada et al. (1978) used SEM to study the ultrastructural features of MLOs. It was concluded that SEM offered a three dimensional visualization of the spatial arrangement and configuration of single organisms, as well as colonies. SEM was used by Maramorosch and Phillips (1981) to study aster yellows and cactus spiroplasmas. Individual aster yellows spiroplasmas appeared rounded and the colonies consisted mainly of spherical cells. Colonies were often linked by thin mycelium-like threads.

Klainer and Pollack (1973) compared SEM with TEM. SEM has the advantage of allowing examination of populations of intact cells in three-dimensional perspective, but is limited in resolution. It provides visualization of surface structure only; the use of TEM remains paramount for examination of intracellular structure.

III. Materials and Methods

A. Test plants

Catharanthus roseus plants (formerly Vinca rosea) having the following Mollicutes-associated diseases were studied : potato witches'-broom (PWB), clover proliferation (CP) (Chen and Hiruki, 1975, 1978a), Eastern aster yellows (EAY, a New York isolate) and Western aster yellows (AY27, an Alberta isolate) (Chen and Hiruki, 1977, 1978b). Healthy C. roseus plants were wedge-grafted with scions from C. roseus plants with the above-mentioned diseases. As control, healthy C. roseus plants were used as scions for grafting. Plants were maintained in the greenhouse at 17°C.

B. Fluorescence microscopy

The effect of buffer systems on the efficacy of DAPI stain

Samples were taken, immediately before examination, from young internode regions of an EAY-diseased C. roseus plant. Free-hand sections were made and fixed in 5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.0 for 2 h. After rinsing in the same buffer, the sections were stained in a solution of DAPI (Sigma) (1 μg/ml) for 20 min. Six sections were stained with each DAPI solution. The solvents used were: distilled water, 0.1 M phosphate buffer (Na_2HPO_4-NaH_2PO_4), at pH values of 5,6,7,8,9, 0.1 M borate buffer ($Na_2B_4O_7.10H_2O$-H_3BO_3-NaCl), at pH values of 5,6,7,8,9, 0.1 M

TRIS buffer ($(CH_2OH)_3CNH_2$), at pH valuesof 6,7,8,9 and phosphate buffer, pH 7 at concentrations of 1 M, 0.5 M, 0.1 M, 0.05 M and 0.01 M. As control, 6 sections from a healthy C. roseus plant were stained in the aqueous DAPI solution. The sections were rinsed in the original buffer and examined in a Zeiss universal fluorescence microscope (HBO 200) with barrier filter 50 and exciter filters BG12 and BG3.

Demonstration of DAPI fluorescence in phloem of plants infected with MLOs.

Plants were tested at weekly intervals beginning 2 weeks after grafting. Samples were taken immediately before examination from petioles of young leaves or from young internode regions of shoots from the stocks of test and control plants maintained in the greenhouse. Free-hand sections about 20-30 μm thick were made within 5-10 min after excising shoots. For staining with DAPI, the sections were fixed in 5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.0 for 2 h. After rinsing in the same buffer, the sections were stained in a solution of DAPI (1 μg/ml) for 20 min (Hiruki, 1981).

C. Immunofluorescence (IF)

Preparation of immunogen

The 'membrane fraction' (MF) from healthy C. roseus and from C. roseus infected with MLO causing PWB, CP, EAY and

AY27 were prepared for use as immunogens as described by Clark et al. (1983).

Leaves of C. roseus were washed for several hours with running tap water to remove soil and other contaminants. The tissue (20 g) was shaken dry, passed through a meat grinder, extracted with ice-cold buffer (1 g/5 ml buffer) and squeezed through cheesecloth. The buffer used was 0.3 M glycine-sodium hydroxide, pH 8.0, with 0.02 M magnesium chloride.

The extract was clarified by centrifugation at 2,000 g for 20 min. The clarified extract was then centrifuged at 39,000 g for 45 min and the large green pellet was resuspended in the original volume of buffer overnight. The next day the extract was clarified by passing the suspension through Whatman No. 2 filter paper in a Buchner funnel. A further cycle of differential centrifugation was applied and the resulting pellet was resuspended in 5 ml of buffer. The suspended material was stored in 0.7 ml aliquots at -18°C and was used as immunogen and antigen in serological tests.

Preparation of antiserum

Antisera to MF preparations of CP, PWB, EAY and AY27 MLO-infected and healthy C. roseus plants were raised in rabbits. Rabbits were injected at 2 week intervals with a 1:1 (v/v) mixture of the immunogen with either Freund's complete (first injection) or incomplete (subsequent injections) adjuvant. One milliliter of the homogenized

mixture was injected at multiple sites involving sub-cutaneous, intra-dermal and intra-muscular injections. Animals were bled (10 ml) at 2 week intervals, beginning 6 weeks after the primary injection. When the appropriate titer of antiserum was obtained, the rabbit was exsanguinated.

The appropriate titer of antiserum was determined by double immunodiffusion. The procedure carried out was a modified version of that described by Kenny (1983). The agar gel composition used was 0.5% agarose in 0.02 M NaCl - 0.01 M phosphate buffer, pH 7.0. Fifteen milliliters of the melted agar were poured into a petri plate. After solidification, 2 sets of holes (diam 3 mm) were cut and emptied of agar by vacuum-suction. There were 7 holes per set; one in the center and 6 around it. The holes were able to hold 15 μl of liquid and were 3 mm apart. Fifteen microliters of antigen were placed in the center hole. Serially diluted antiserum (15μl) was placed in the surrounding holes (1, 1/2, 1/4, 1/8, 1/16, 1/32) as well as an initial check of the pre-immune serum. As control, healthy antigen replaced the test antigen in the center hole of the second set. The petri plate was incubated at room temperature in a moist chamber for 2-3 days. Plates were observed for precipitin lines.

The blood obtained from the exsanguinated rabbit was incubated at room temperature for 4 h until it clotted. The plasma was collected with a Pasteur pipette and clarified by

centrifugation at 5,000 g for 30 min. Sodium azide at a concentration of 0.02% (w/v) was added to the clarified plasma.

To remove the antibodies to healthy plant proteins, the antisera were cross-absorbed with acetone-extracted powder of healthy C. roseus. For every 10 ml of antiserum, 10 g of healthy leaf material was used for preparing acetone-extracted powder. The healthy leaf material was passed through a meat grinder and extracted in 200 ml of acetone. The extract was filtered through a Buchner funnel and Whatman No. 4 filter paper. The powder was washed several times with acetone (750 ml total) until it was almost colourless and placed in a beaker or wide-mouth conical flask. One hundred and twenty milliliters of 80% ethanol were added. The flask was placed in a boiling water bath and boiled for 15 min. The plant protein powder was then washed with 750 ml of PBS, placed in a test tube with 10 ml of antiserum and incubated at 40°C for 2 1/2 h. After leaving overnight at 4° C, the mixture was centrifuged at 17,000 g for 15 min. The supernatant containing the antiserum was collected.

The immunoglobulin fraction of the antiserum was precipitated with ammonium sulphate (Clark and Adams, 1977). Equal volumes of antiserum and saturated ammonium sulphate were mixed together and incubated at room temperature for 90 min. The mixture was centrifuged at 3,000 g for 10 min and the precipitate resuspended in 2 ml of 0.005 M PBS, pH 7.5.

The resuspension was placed in dialysis tubing and dialysed overnight at 4°C against two changes of approximately 2 liters 0.005 M PBS.

The concentrations of the purified immunoglobulins were determined by measuring the absorbance at 280 nm (A_{280}) of a 1/20 dilution of immunoglobulin and using the formula (Johnstone and Thorpe, 1982)

$$\text{Conc (mg/ml)} = \frac{A_{280} \times 10 \times 20}{13.5}$$

The immunoglobulins were then diluted with PBS to 10 mg/ml and stored in 1 ml aliquots at -18°C.

Indirect IF

The IF staining procedure described by Kawamura (1977) was modified to provide a simple and rapid method which is suitable for use with thick plant sections. In this investigation the following method was used. Free-hand 30-60 µm sections from the internode region were cut and placed in a 1.5 ml micro-centrifuge tube. Two hundred microliters of 95% ethanol were added and the sections were fixed for 5 min. The sections were then washed three times with 200 µl PBS. Fifty microliters of the immunoglobulin were placed in the tube for 15 min at room temperature. The sections were washed as above. Fifty microliters of labeled antirabbit IgG FITC conjugate (Sigma) were added to the sections in the tube and incubated for 15 min at room temperature. The sections were washed as above. The final

washing was replaced by 200 µl of the mounting medium, 1:1 PBS and glycerol. The sections were mounted on glass slides with coverslips and viewed under a fluorescence microscope.

Immunoglobulins obtained against the membrane fraction of PWB, CP, EAY and AY27 diseased plants were used to test sections from plants with the four diseases as well as sections from healthy control plants.

D. Electron microscopy

At the time of sectioning for staining with DAPI, sections were made from the same region for use in electron microscopy studies. Tissue pieces about 1 mm thick were fixed for 3 h in 3% glutaraldehyde in 0.1 M phosphate buffer, pH 7.0. The tissue was washed in the same buffer, postfixed in 2% osmium tetroxide for 3 h and subsequently washed in the buffer. The tissue samples were dehydrated in a graded ethanol series, 70%, 90%, 95%, 95%, 95% for 20 min in each.

Transmission electron microscopy (TEM)

The dehydrated samples were then placed in two changes of propylene oxide for 20 min each. The samples were embedded in Araldite 502 and sectioned 50-90 nm thick with an ultramicrotome equipped with a glass knife. The sections were stained with uranyl acetate and lead citrate and examined under a Philips 201 electron microscope at 60 kV.

Scanning electron microscopy (SEM)

The dehydrated samples were dried at critical point in CO_2, mounted on SEM stubs with silver conducting paste and sputter coated with approximately 15 nm of gold. Sections were examined under a Cambridge Stereoscan 250 or 100 electron microscope.

In further studies, preparation of tissue for SEM observation involved the fixation of 3-4 cm of stem tissue in 3% glutaraldehyde in 0.1 M phosphate buffer, pH 7.0 overnight before hand sectioning. The tissue was rinsed, fixed in 2% osmium tetroxide in water and rinsed again as before and dehydrated in the same ethanol series or in 35%, 50%, 70%, 95%, 95%, 95% ethanol for 10 min in each. The sections were dried at critical point in CO_2 and processed as before.

Paraffin embedding of tissue for SEM observation was also carried out. Using the procedure described by Jensen (1962) as a guideline, the length of time recomended for the steps were considerably shortened due to the thinness of the sections prepared. The stem tissue was fixed in 3% glutaraldehyde overnight before hand sectioning. The tissue was rinsed, fixed in 2% osmium tetroxide for 2 h and rinsed again. The dehydration was carried out using H_2O, ethanol and tertiary butyl alcohol (TBA) in the following ratios: i) 70:30:0, ii) 50:40:10, iii) 30:50:20, iv) 15:50:35, v) 0:45:55, vi) 0:25:75 for 10 min in each and in three changes of 100% TBA for 15 min in each. The sections were then

placed in 1:1 TBA:paraffin in a capped tube overnight in a 60°C oven. The next day the cap was removed. After 3 h the sections were placed in three changes of paraffin for 4 h in each in the 60°C oven. The sections were embedded in paraffin by placing them in molten paraffin and cooling down to room temperature. On solidification, the paraffin blocks were sectioned with a rotary microtome to produce 50μm thin sections. The sections were placed on round coverslips that exactly fitted onto SEM stubs.

The coverslip was first coated with a thin layer of Haupt's adhesive, made by dissolving 1 g gelatin in 100 ml water at 90°C, cooling to 30°C, adding 15 ml glycerine and 2 g of phenol and filtering. A drop of water was placed on the coverslip and the sections were floated on the water. The coverslip was transferred to a slide warmer at 45°C to allow the tissue to stretch. When the tissue was fully extended, the coverslip was removed from the slide warmer and allowed to cool. The excess water was removed from the coverslip. To remove the paraffin from the sections, the coverslip was placed in xylene, in a 1:1 mixture of xylene and ethanol and then in ethanol for 5 min in each. The coverslip was dried, mounted onto SEM stubs with silver conducting paste and processed as before.

IV. Results

A. The fluorochrome DAPI

Buffer systems

Results showed that the different buffer systems had no effect on the efficacy of DAPI as a stain. DAPI solutions of 1 μg/ml in distilled water, 0.1 M phosphate buffer, pHs 5 to 9, 0.1 M borate buffer, pHs 5 to 9 and 0.1 M TRIS buffer, pHs 6 to 9, as well as DAPI solutions of 1 μg/ml in phosphate buffer, pH 7 at concentrations of 1 M, 0.5 M, 0.1 M, 0.05 M and 0.01 M, all gave positive DAPI fluorescence in the phloem of EAY diseased C. roseus plant sections. Healthy plant sections exhibited no DAPI fluorescence in the phloem after DAPI staining. This showed that the type of buffer, its pH and concentration all had little effect on the efficacy of DAPI. Since no difference was found with the different buffers, an aqueous DAPI solution was used in the following demonstration of DAPI fluorescence in the phloem of infected plants.

Staining of infected plants

Fluorescence in the phloem of plant sections stained with DAPI was correlated to the symptoms observed in the shoot. Table 2 shows a summary of the results obtained in this study. Twenty plants were grafted for each disease and for the control. Not all plants had shoots in the stock

Table 2 : Diagnosis of MLO infection in shoots of Catharanthus roseus stocks onto which scions infected with MLOs of Eastern aster yellows, New York isolate (EAY), Western aster yellows, Alberta isolate (AY27), potato witches'-broom (PWB) and clover proliferation (CP) were grafted. As control, healthy scions were grafted onto the stocks.

	W2[1]		W3		W4		W5		W6		W7	
sample	**S[2]**	**D[3]**	**S**	**D**	**S**	**D**	**S**	**D**	**S**	**D**	**S**	**D**
Healthy	0	0	0	0	0	0	0	0	0	0	0	0
EAY	0	0	22	22	50	50	80	60	88	75	86	100
AY27	0	8	27	64	33	83	60	60	50	75	100	100
PWB	0	9	8	0	10	40	57	43	17	67	50	83
CP	0	0	0	16	0	80	28	86	20	100	75	100

1) W2=2nd week after grafting
2) S=symptoms; percentage positive (out of 3 to 14)
3) D=DAPI; percentage positive (out of 3 to 14)

region every week; the total number of plants tested for each disease ranged from 3 to 14, while for the control the range was 3 to 12. A positive result was considered to be DAPI fluorescence in the phloem (Figs. 1a,b,c,d). One fluorescent spot constituted a positive result. No DAPI fluorescence in the phloem was observed in healthy plants (Fig. 2). The determination of positive symptoms, particularly at early stages of infection, was more difficult to discern. New shoots, often less than a week old, were too small to show symptoms. Only obvious symptoms were considered positive.

B. Immunofluorescence (IF)

MLO infections were detected by the indirect IF method. The use of 'membrane fraction' preparations from MLO-infected plants as immunogens produced antisera in rabbits. Titers of the antisera were determined by double immunodiffusion. These ranged from 1/4 to 1/16 (Figs. 3a,b,c,d). IF was positive with MLO-infected plant sections and negative with healthy plant sections (Table 3). All four antisera (EAY, AY27, PWB, CP) gave positive results with all the MLO-infected plant sections (EAY, AY27, PWB, CP) (Figs. 4Aa to Dd). Stronger fluorescence was observed with EAY and AY27 plant sections than with PWB and CP plant sections. This corresponds with the observations that EAY and AY27 have stronger symptoms than CP and PWB. Healthy plant sections tested with antisera against EAY, AY27, PWB

Table 3 : Indirect immunofluorescence observed with cross-sections of Catharanthus roseus stems infected with MLOs of Eastern aster yellows, New York isolate (EAY), Western aster yellows, Alberta isolate (AY27), potato witches'-broom (PWB) and clover proliferation (CP). As control, healthy stem sections were tested.

Plant tissue[1]	Antiserum			
	EAY	AY27	PWB	CP
Healthy	-	-	-	-
EAY	++++	++++	++++	++++
AY27	++++	++++	++++	++++
PWB	+	+	+	+
CP	+	+	+	+

1) Three stem sections were used in each test
- = no immunofluorescence in the phloem
+ = weak immunofluorescence in the phloem
++++ = strong immunofluorescence in the phloem

and CP showed no fluorescence in the phloem region (Fig. 5).

C. Electron microscopy

TEM

DAPI results were compared with TEM thin sections (Table 4). MLOs were observed in thin sections of diseased plant tissue from shoots that had given positive results with DAPI (Figs. 6a,b,c,d). In sections made the previous week from shoots of the same plant where DAPI results were negative, MLOs were observed in two of the four diseased plants, AY27 and CP (Figs. 7a,b). No MLOs were observed in the healthy control.

SEM

Sections made from shoots that were tested with DAPI and observed by TEM were observed by SEM. MLO bodies were observed in both sections that had given positive DAPI results as well as those that had given negative DAPI results (Figs. 8a to h and 9a to h). No MLO bodies were observed in healthy controls (Figs. 10a,b).

Fixation of the plant tissue in 3% glutaraldehyde overnight before hand sectioning made observation of MLOs easier because fewer amyloplasts were observed in the area of the phloem elements. Dehydrating tissue in the ethanol series starting at 70% resulted in collasped or crushed phloem cells while tissue dehydrated in the ethanol series

Table 4 : Comparison of DAPI with TEM for the detection of MLOs in stem sections of Catharanthus roseus infected with MLOs of Eastern aster yellows, New York isolate (EAY), Western aster yellows, Alberta isolate (AY27), potato witches'-broom (PWB) and clover proliferation (CP). As control, healthy stem sections were examined.

Plant tissue	Weeks after grafting	Symptoms	DAPI[1]	TEM[2]
Healthy	3	-	-	-
EAY	3	-	-	-
EAY	4	+	+	+
AY27	2	-	-	+
AY27	3	-	+	+
PWB	7	-	-	-
PWB	8	-	+	+
CP	3	-	-	+
CP	4	-	+	+

1) Six sections were examined
2) Two sections were examined
- = symptoms; no symptoms observed
DAPI; no DAPI fluorescence in the phloem observed
TEM; no MLOs obseved
+ = symptoms; symptoms observed
DAPI; DAPI fluorescence in the phloem observed
TEM; MLOs observed

starting at 35% had better preserved phloem cells (Figs. 11a to e). No MLOs were found in the healthy tissue thus processed. Sections embedded in paraffin and sectioned by rotary microtome showed well-preserved cells (Figs. 12a,b). Some tissue was damaged or torn during the process. No MLOs were observed in the 10 sections examined from C. roseus plants infected with MLOs of Eastern aster yellows, New York isolate. MLOs were detected from the same tissue prepared by fixing overnight in 3% glutaraldehyde and dehydrated in the ethanol series starting at 35% (Figs. 11d,e).

V. Discussion

With the enhancement of new monitoring agents at the molecular level, anatomical studies of cellular and subcellular structures by light and electron microscopy of healthy and diseased plants have brought about significant advances in plant pathology. Increasing numbers of new plant pathogens demand diagnostic procedures that provide higher specificity, rapidity and simplicity. In this study, four diagnostic techniques involving light and electron microscopy were investigated.

A. DAPI as a diagnostic stain

Achievements of the present investigation are twofold. Firstly, my study of the effect of different buffer systems on the efficacy of DAPI in MLO infected plants showed that the fluorochrome is not sensitive to the ionic strength and pH of the medium. Masotti et al. (1982) reported that no fluorescence change is detected in the emission intensity of the DAPI-linear DNA complex in the presence of salts. They disputed the theory that DAPI binds to DNA non-intercalatively by an electrostatic attachment of the cationic molecules along the negatively charged backbone of DNA. If this were the case, the fluorescence enhancement should be sensitive to the ionic strength and pH of the medium. DAPI was shown to bind to DNA intercalatively (Schweizer, 1976; Chandra and Mildner, 1979). Coleman et al. (1981) examined the effects of pH and salts on the

fluorescence intensity of DAPI and showed a broad plateau of DAPI fluorescence between pH 4 and 8 which did not require any cation. Fluorescence was, however, severely attentuated at pH 2.

Secondly, results of this study showed that MLOs from four different diseases could be detected with DAPI as early as 2 weeks after grafting, before the development of visible symptoms. The detection rates by DAPI were generally higher than those by observation of symptoms. TEM results revealed the presence of MLOs in shoots that had negative results with DAPI the week prior to positive staining of shoots from the same plants. It is assumed that with DAPI staining, a certain concentration of MLOs is required in a given region for enough DAPI binding to occur which would result in observable fluorescence. Thus the week before fluorescence was observed, MLOs were present in the shoots at a lower concentration and not detected by DAPI. In all but one case recognizable symptoms were not yet developed. Although not always sensitive to mild or early infections, DAPI can be useful as a diagnostic stain.

B. Immunofluorescence (IF)

The combination of immunological and histological approaches is a promising line of research which has proved extremely useful for histopathology in this investigation. The purpose of the present immunohistological procedure is the identification of MLOs in diseased plant tissues.

Antibodies produced against purified immunogens possess a high degree of specificity towards antigenic determinants. Since an antibody molecule binds with its antigenic determinant in a specified range of reactivity, immunochemical methods are one of the most sensitive techniques in molecular biology and histopathology. The indirect IF technique was modified for use with thick sections of C. roseus in this study. In the medical field, one standard method makes use of glass slides onto which the unknown antigen is smeared (Kawamura, 1977). The addition of primary and secondary antiserum is carried out directly on the slide. Rao et al. (1978) used the IF technique to detect clover yellow mosaic virus (CYMV) inclusion bodies in cowpea. Epidermal strips were peeled off the leaf and adhered to a glass slide with Haupt's adhesive. In the study described here, free-hand sections were cut. These sections were too thick (30-60 μm) and therefore too heavy to be adhered by Haupt's adhesive onto a glass slide. Thorough washing of the slide would result in the tissue sections being washed off. The sections were therefore placed in micro-centrifuge tubes and all solutions added to and removed from the tube with a Pasteur pipette. Advantages of this method were that the sections were totally immersed in the solutions and that smaller quantities of antiserum were required (50μl each time). Kawamura's procedure required the smear to be covered with solutions, i.e. a considerably larger volume. It was also

found that incubation times could be shortened. The entire modified procedure took just three hours for the processing of 5 different tissues with 4 antisera (i.e. 20 tests). Kawamura's method requires incubation times of 30-60 min each and washings of 15 min each.

With the modified technique, incubation could be carried out at room temperature. Moist chambers were not required as the solutions in the micro-centrifuge tubes would not dry up, especialy when the tubes were capped. Shakers were not required for washing as they are in Kawamura's technique. Thus the modifications provided a method of IF for use with thick plant sections which was both rapid and simple to carry out.

The indirect method of IF was carried out. With this method, only the secondary antibody is conjugated with the fluorochrome. This can be used with the primary antibodies specific for MLOs. The secondary antibody may be purchased commercially.

Results of this study showed that the antiserum produced was capable of detecting MLO antigen in diseased plant tissue. All four antisera tested showed similar results with the four MLOs. Healthy tissue showed no fluorescence. This is the first report of the successful use of indirect IF in detecting plant MLOs.

C. Electron microscopy

TEM

TEM has been used to observe plant MLOs since their discovery in 1967. In this study, typical MLOs were found in thin sections of severely diseased plants which had given positive results with the DAPI stain. Shoots from the previous week when DAPI results had been negative, were also examined by thin sectioning and in two cases MLOs were observed. Thus TEM is the more reliable method of MLO detection.

SEM

SEM studies revealed cytoplasmic structures resembling MLOs in the phloem regions of diseased tissue which fluoresced when stained with DAPI. In initial tests, the sections were made before fixing in 3% glutaraldehyde. This resulted in the observation of large numbers of amyloplasts on the surface of the sections. Zimmermann (1978) reported that as sieve tubes are normally under high positive pressure (of the order of 10-20 bars), they exude at least very briefly when punctured. Surging movements due to pressure release can cause artifacts and often make it difficult to visualize what the structure might have been before the pressure surge. The amyloplasts are exuded from the cells due to this pressure surge. Thus MLOs may be difficult to detect because of the presence of the exudates

on the surface of the section. These exudates are much larger than the MLOs.

When fixation of the plant tissue before sectioning was carried out, the initial problem of pressure surging was eliminated. Fewer amyloplasts were observed on the surface of the section. Embedding sections in paraffin also eliminated pressure surging. No structures resembling MLOs were observed, however, in the diseased tissue examined. Thus SEM appears to be of limited use in detecting MLOs in plant tissue. Overnight fixation before sectioning, followed by a gradual dehydration series, is recommended.

D. Evaluation of diagnostic techniques investigated

Four diagnostic techniques involving light and electron microscopy were investigated. Both levels of microscopy can provide useful information. Electron microscopy provides information about MLOs and their environment at the ultrastructural level. Visualisation of MLOs at high magnifications is vital for definitive diagnosis of plant diseases. In light microscopy, however, extensive areas of tissue in a large field of view can be examined, assuring adequate sampling for diagnistic work. This is especially important in plant mycoplasmology where MLOs are unevenly distributed in the phloem.

The DAPI stain is suitable for use in diagnosis. The technique is rapid and large numbers of samples may be processed easily. The indirect IF test was also found to be

a useful technique for diagnosis. It is capable of detecting the specific fluorescence of the MLO antigen. This makes the technique more specific than the DAPI technique, with which nuclei and mitochondria as well as MLOs fluoresce. In some cases, the strong DAPI fluorescence in plant nuclei may make interpretation of results impossible. TEM, although time consuming, is the most reliable technique for the identification of MLOs, yielding more accurate diagnostic information than the DAPI technique for detecting MLOs in mild or early infections. SEM studies showed that there is a potential for the use of scanning electron microscopy to detect plant MLOs. Further work is required to develop a useful procedure.

VI. Figures and Legends

Figure 1 :
Cross-sections of diseased Catharanthus roseus stems or petioles showing DAPI fluorescence in the phloem
a) Eastern aster yellows, New York isolate (EAY) (120 X)
b) Western aster yellows, Alberta isolate (AY27) (120 X)
c) potato witches'-broom (PWB) (120 X)
d) clover proliferation (CP) (120 X)

X=xylem, (arrow)=phloem
Bar is 0.1 mm.

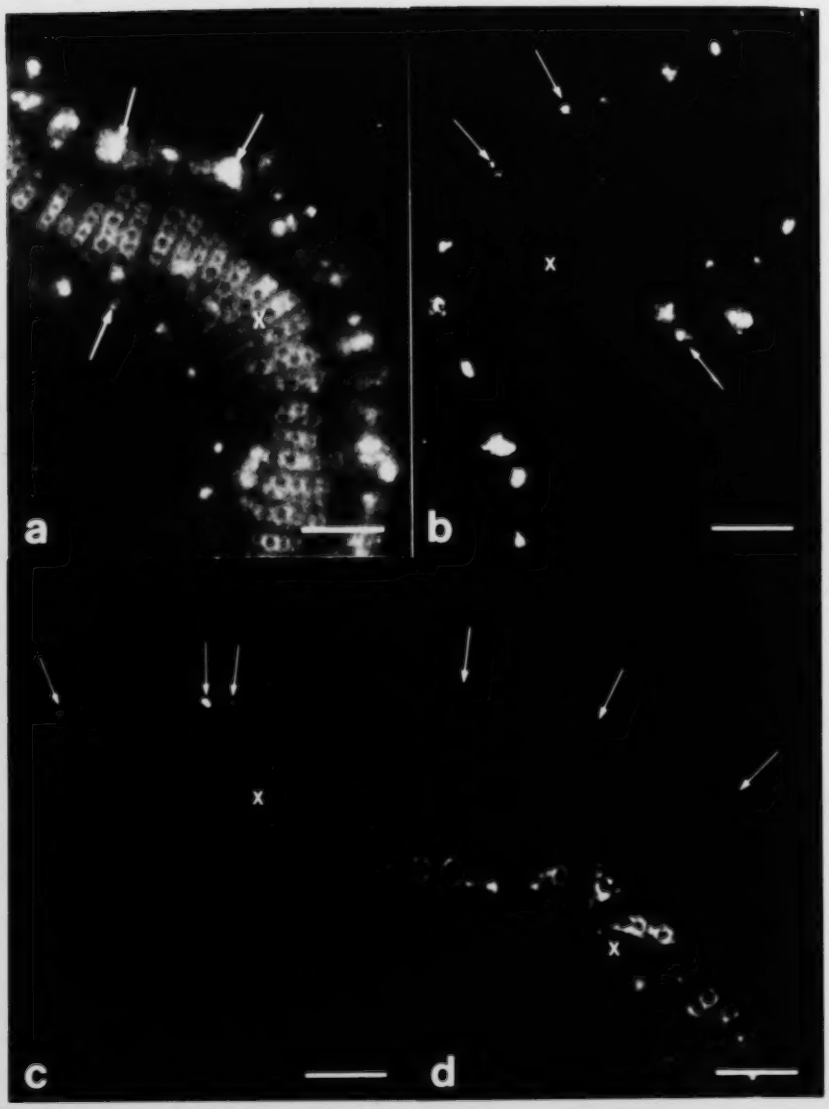

a
b
c
d

Figure 2 :
Cross-section of healthy Catharanthus roseus stem stained with DAPI showing no fluorescence in the phloem (230 X)

X=xylem, (arrow)=phloem
Bar is 0.1 mm.

X

Figure 3 :
Titers of antisera produced against MLOs of
a) Eastern aster yellows, New York isolate (EAY) (1/16)
b) Western aster yellows, Alberta isolate (AY27) (1/16)
c) potato witches'-broom (PWB) (1/4)
d) clover proliferation (CP) (1/16)
These titers were determined by double immunodiffusion tests.

Ag=antigen

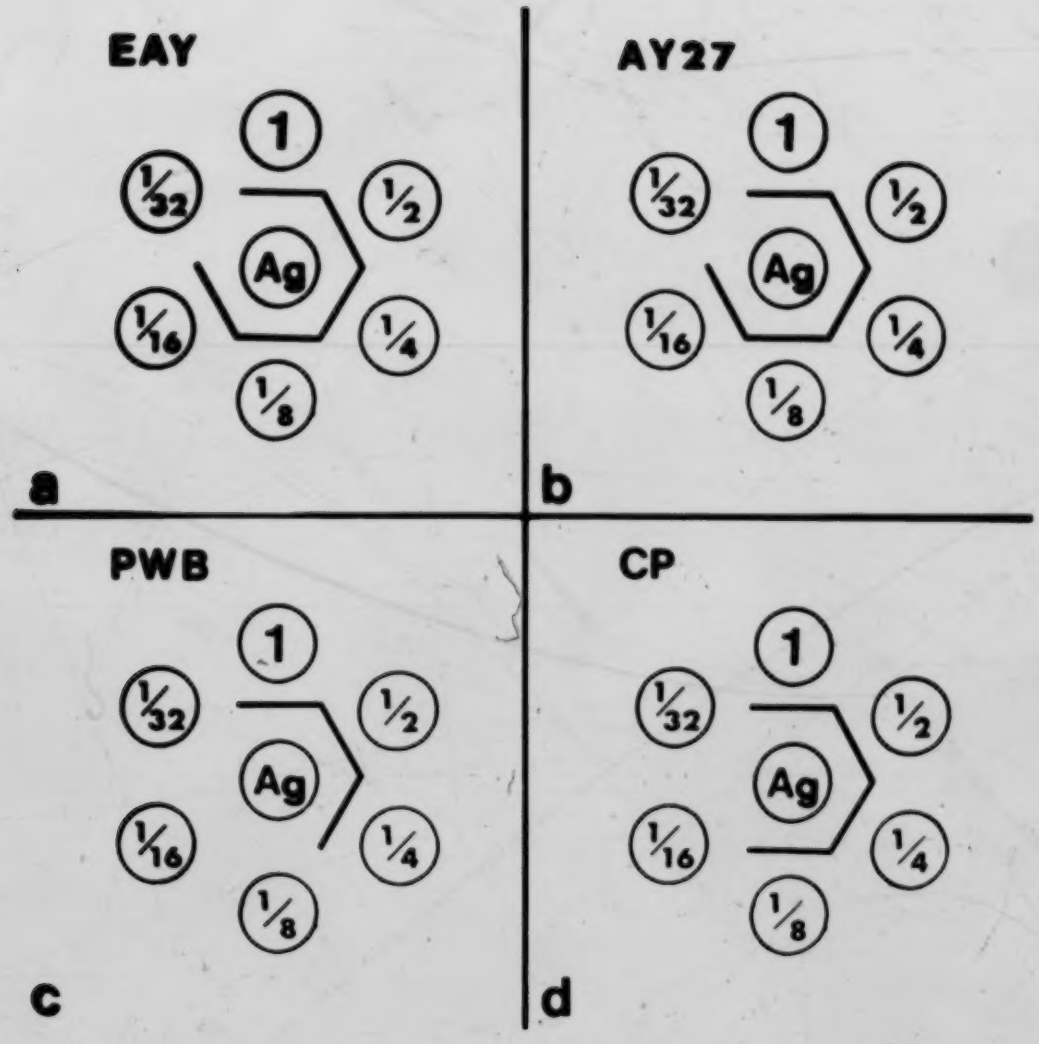

EAY
1
1/32
1/2
Ag
1/16
1/4
1/8
a
AY27
1
1/32
1/2
Ag
1/16
1/4
1/8
b
PWB
1
1/32
1/2
Ag
1/16
1/4
1/8
c
CP
1
1/32
1/2
Ag
1/16
1/4
1/8
d

Figure 4A :
Indirect immunofluorescence;
Stem cross-sections of Catharanthus roseus infected with MLOs of
A) Eastern aster yellows, New York isolate (EAY)
tested with
a) anti-EAY serum
b) anti-AY27 serum
c) anti-PWB serum
d) anti-CP serum

X=xylem, (arrow)=phloem
Aa, 160 X; Ab,c,d, 100 X.
Bar is 0.1 mm.

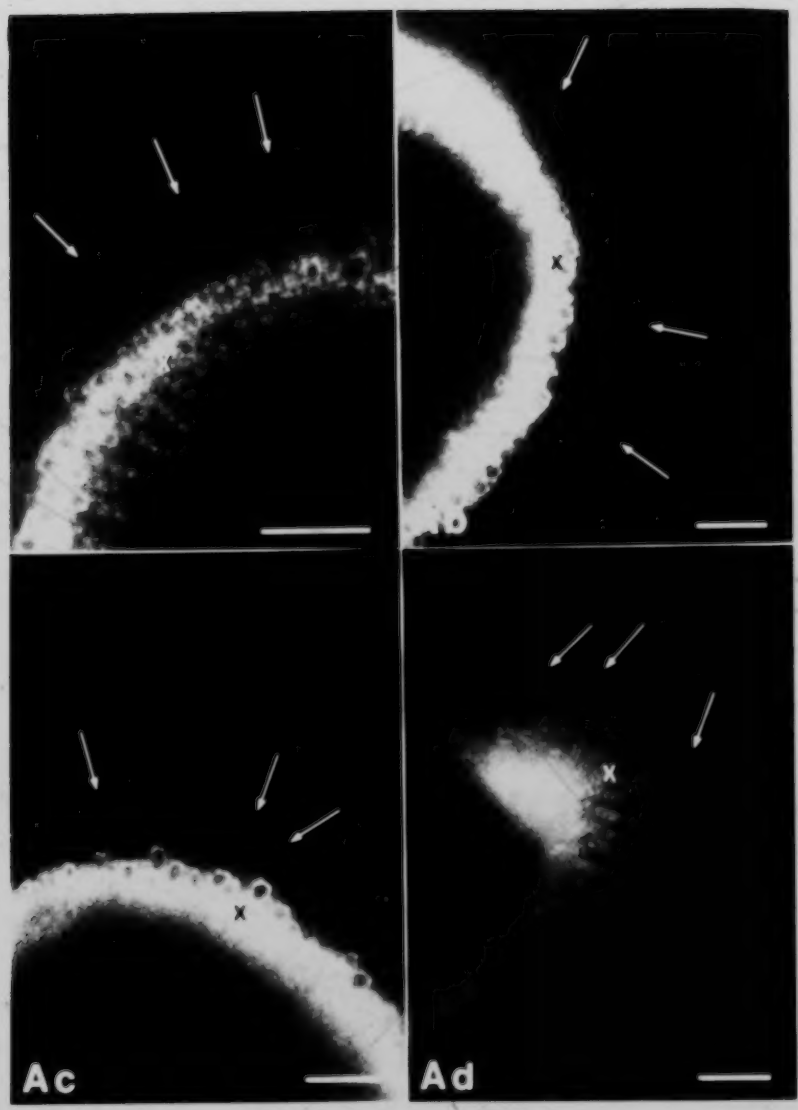

x
x
Ac
Ad

Figure 4B :
Indirect immunofluorescence;
Stem cross-sections of Catharanthus roseus infected with MLOs of
B) Western aster yellows, Alberta isolate (AY27)
treated with
a) anti-EAY serum
b) anti-AY27 serum
c) anti-PWB serum
d) anti-CP serum

X=xylem, (arrow)=phloem
Ba,b,c,d, 120 X.
Bar is 0.1 mm.

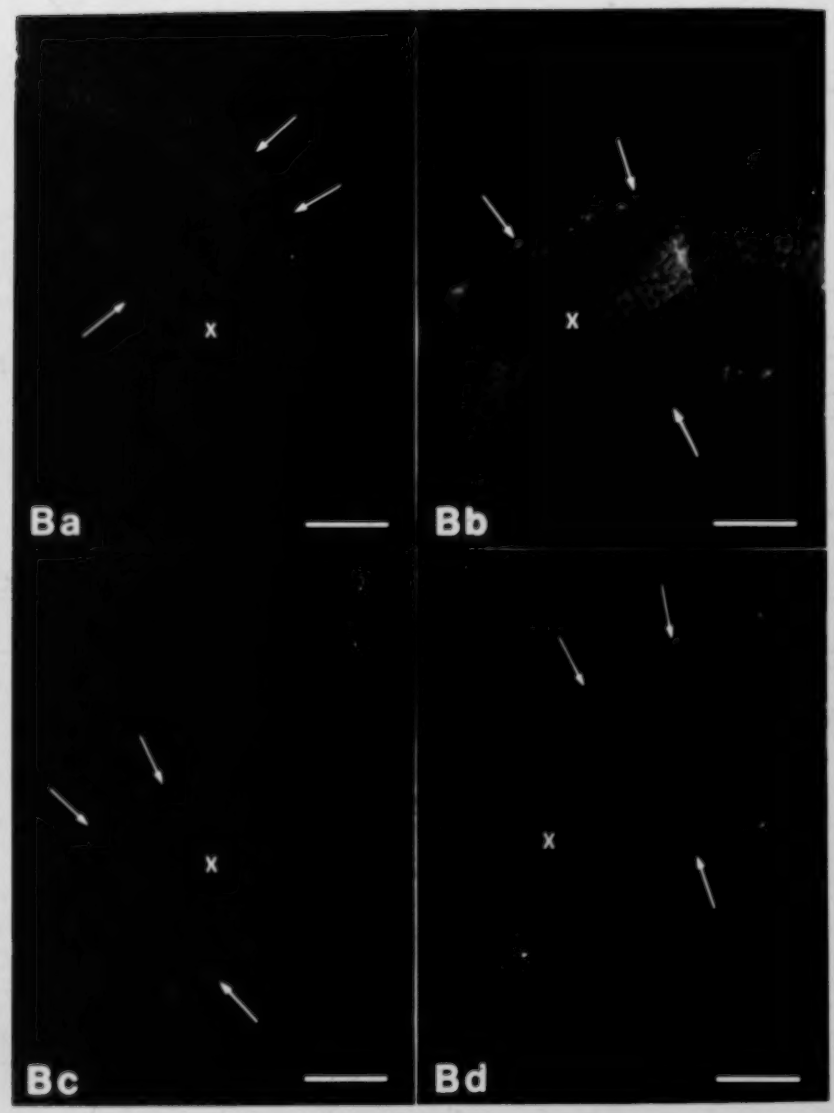

Ba
Bb
Bc
Bd

Figure 4C :
Indirect immunofluorescence;
Stem cross-sections of Catharanthus roseus infected with
MLOs of
C) potato witches'-broom (PWB)
treated with
a) anti-EAY serum
b) anti-AY27 serum
c) anti-PWB serum
d) anti-CP serum

X=xylem, (arrow)=phloem
Ca,b,c,d, 120 X.
Bar is 0.1 mm.

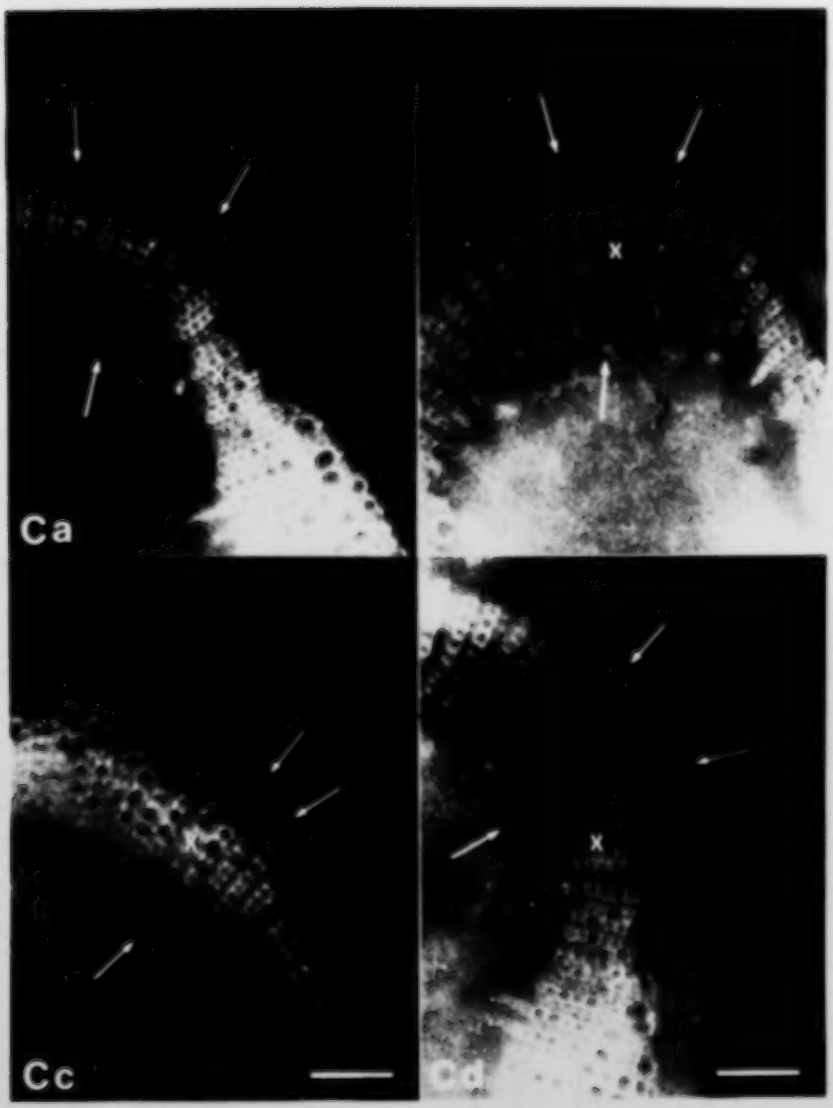

Ca
Cc

Figure 4D :
Indirect immunofluorescence;
Stem cross-sections of Catharanthus roseus infected with
MLOs of
D) clover proliferation (CP)
treated with
a) anti-EAY serum
b) anti-AY27 serum
c) anti-PWB serum
d) anti-CP serum

X=xylem, (arrow)=phloem
Da,b,c,d 120 X.
Bar is 0.1 mm.

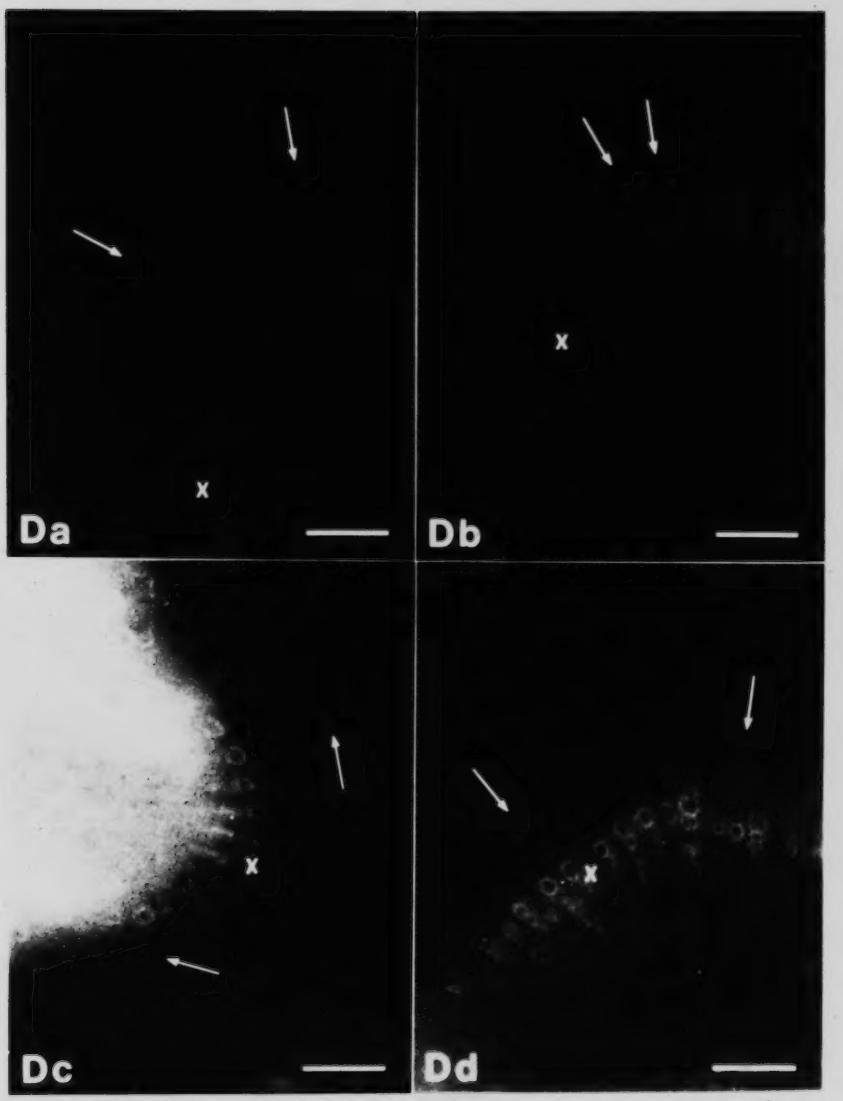

Da
Db
Dc
Dd

Figure : 5
Indirect immunofluorescence control;
Cross-section of healthy Catharanthus roseus stem tested with anti-Eastern aster yellows, New York isolate (EAY) serum (230 X)

X=xylem
Bar is 0.1 mm.

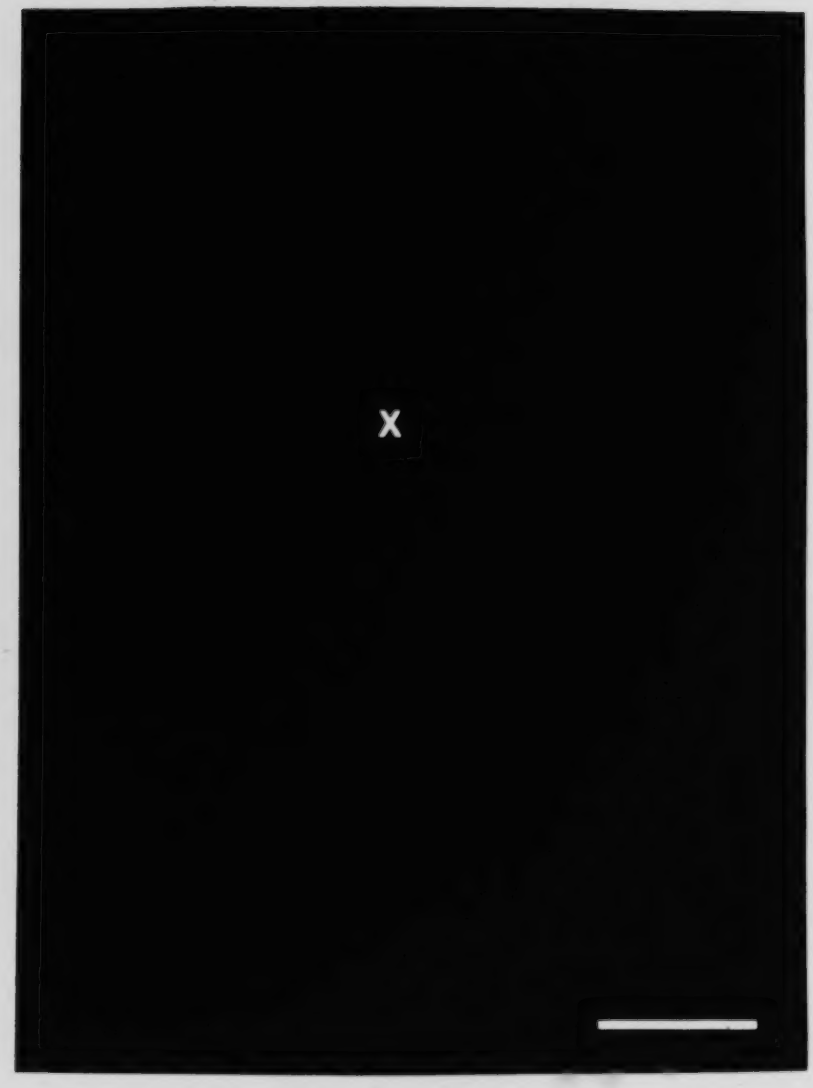

X

Figure 6 :
Transmission electron micrographs of MLOs in the phloem of stem cross-sections of diseased Catharanthus roseus that had given positive DAPI results
a) Eastern aster yellows, New York isolate (EAY) (22,000 X)
b) Western aster yellows, Alberta isolate (AY27) (22,000 X)
c) potato witches'-broom (PWB) (12,500 X)
d) clover proliferation (CP) (16,500 X)

MLO=mycoplasmalike-organism, CW=cell wall
Bar is 1 μm.

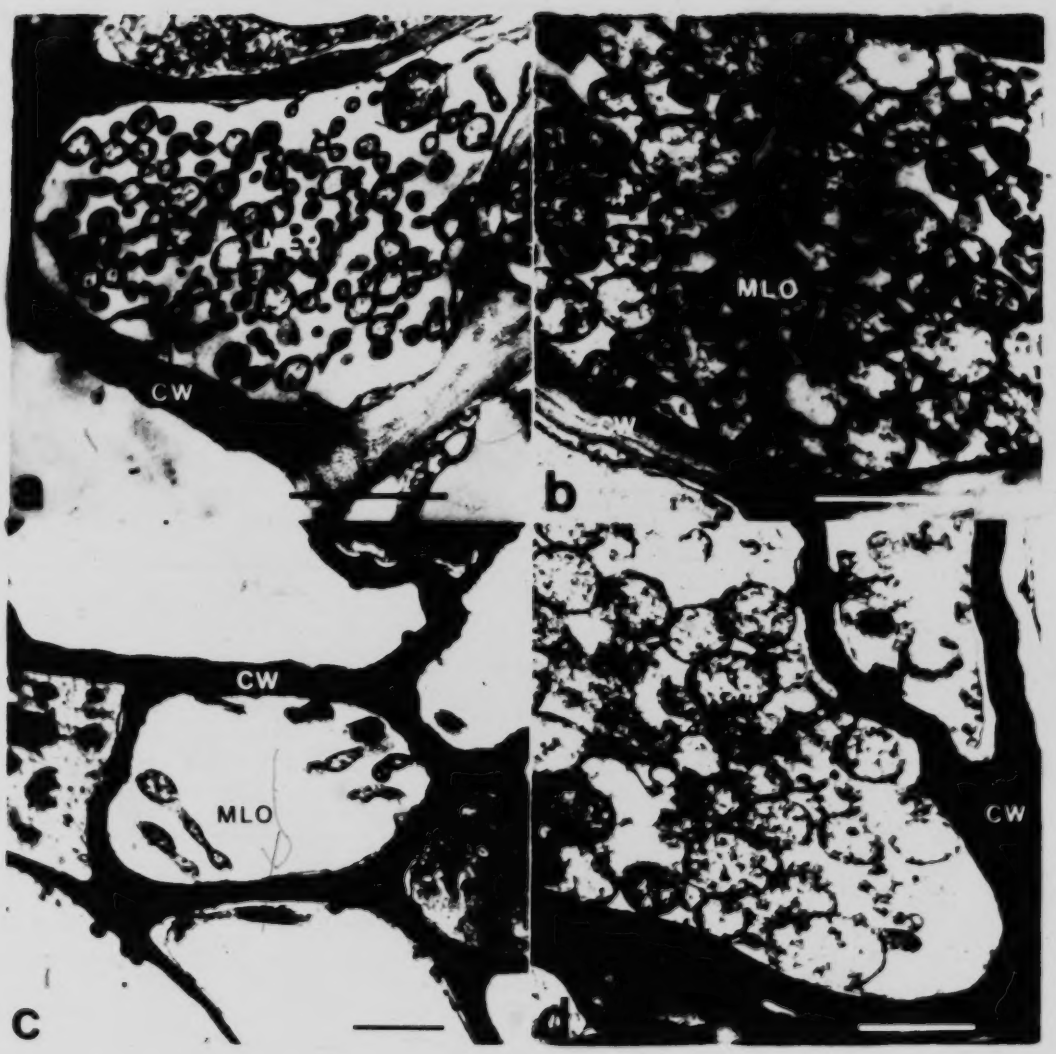

CW
a
MLO
CW
b
CW
MLO
c
CW
d

Figure 7 :
Transmission electron micrographs of MLOs in the phloem of stem cross-sections of diseased Catharanthus roseus that had given negative DAPI results
a) Western aster yellows, Alberta isolate (AY27) (5,700 X)
b) clover proliferation (CP) (24,500 X)

MLO=mycoplasmalike organism, M=mitochondria, CW=well wall
A=amyloplast
Bar is 1 µm.

MLO
a
MLO
b

Figure 8 :
Scanning electron micrographs of structures resembling MLOs in the phloem of stem cross-sections of diseased Catharanthus roseus that had given positive DAPI results
Eastern aster yellows, New York isolate (EAY) a) (2,000 X) b) (8,000)
Western aster yellows, Alberta isolate (AY27) c) (490 X) d) (4,100 X)
potato witches'-broom (PWB) e) (990 X) f) (4,100)
clover proliferation (CP) g) (490 X) h) (8,000 X)

X=xylem, (arrow)=phloem, 'MLO'=structures resembling mycoplasmalike organisms, A=amyloplast

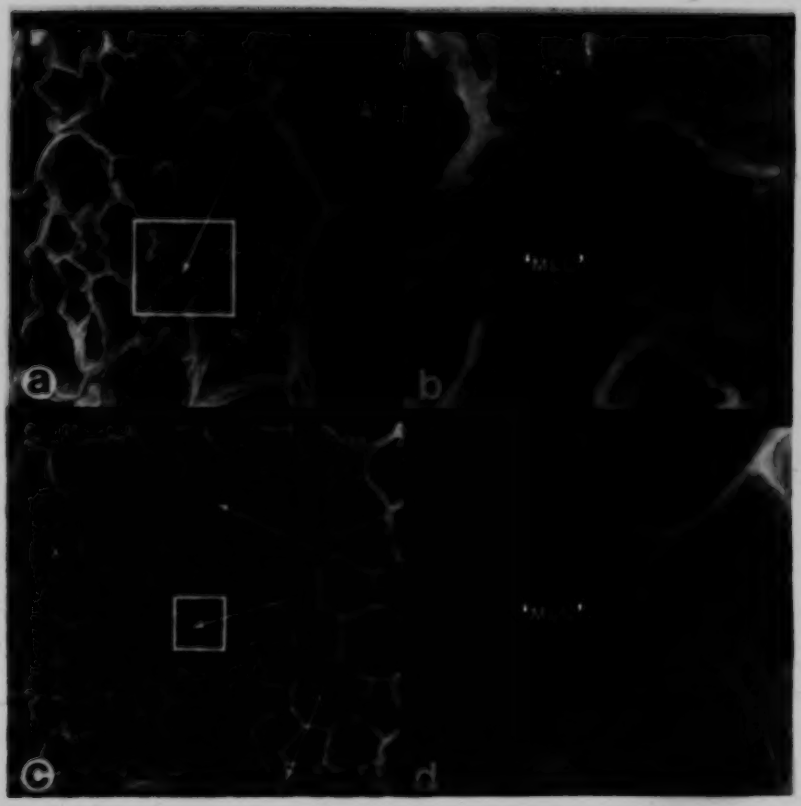

a
b
c
d

Figure 8 :
Scanning electron micrographs of structures resembling MLOs in the phloem of stem cross-sections of diseased Catharanthus roseus that had given positive DAPI results
Eastern aster yellows, New York isolate (EAY) a) (2,000 X) b) (8,000)
Western aster yellows, Alberta isolate (AY27) c) (490 X) d) (4,100 X)
potato witches'-broom (PWB) e) (990 X) f) (4,100)
clover proliferation (CP) g) (490 X) h) (8,000 X)

X=xylem, (arrow)=phloem, 'MLO'=structures resembling mycoplasmalike organisms, A=amyloplast

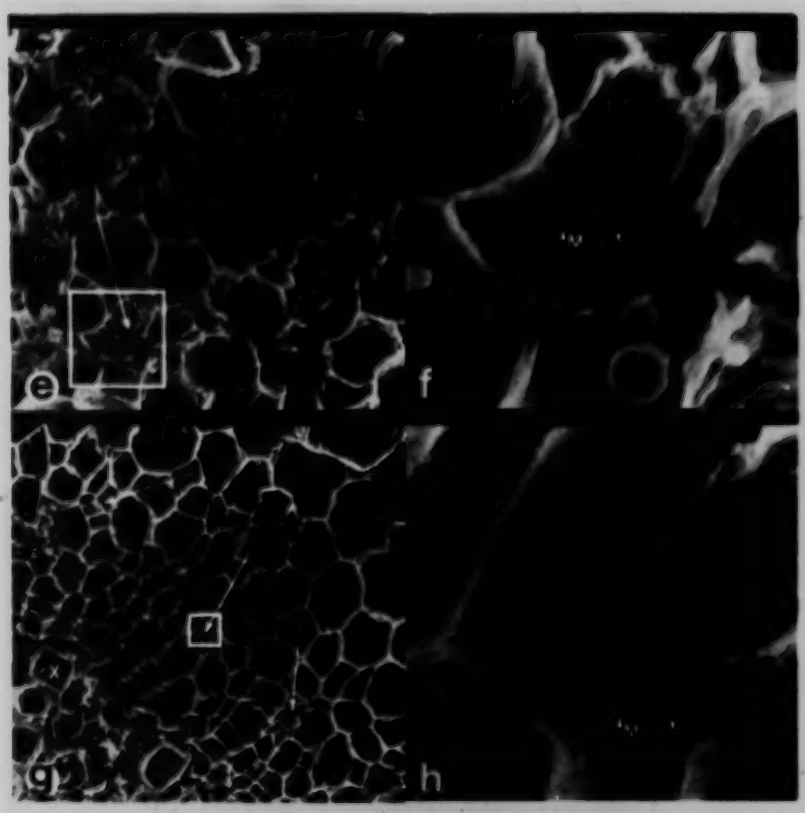

e
f
g
h

Figure 9 :
Scanning electron micrographs of structures resembling MLOs in the phloem of stem cross-sections of diseased *Catharanthus roseus* that had given negative DAPI results
Eastern aster yellows, New York isolate (EAY)a) (350 X) b) (5,800 X)
Western aster yellows, Alberta isolate (AY27)c)(250 X) d) (1,900 X)
potato witches'-broom (PWB) e) (980 X) f) (2,020 X
clover proliferation (CP) g) (480 X) h) (8,000 X)

X=xylem, (arrow)=phloem, 'MLO'=structures resembling mycoplasmalike organisms, A=amyloplast

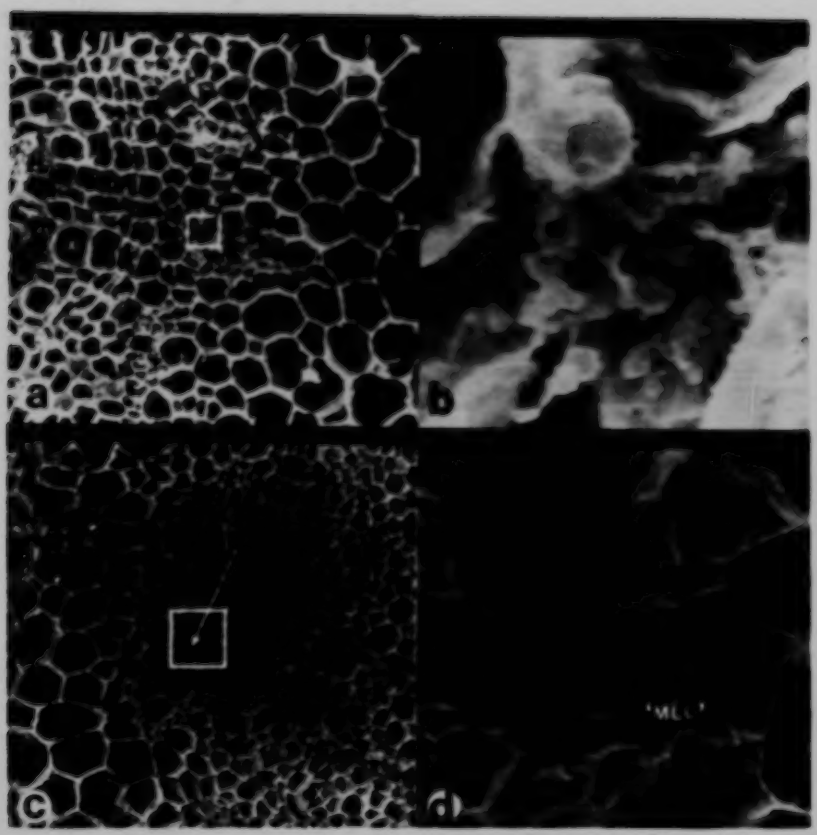

a
b
c
d

Figure 9 :
Scanning electron micrographs of structures resembling MLOs in the phloem of stem cross-sections of diseased Catharanthus roseus that had given negative DAPI results
Eastern aster yellows, New York isolate (EAY)a) (350 X) b) (5,800 X)
Western aster yellows, Alberta isolate (AY27)c)(250 X) d) (1,900 X)
potato witches'-broom (PWB) e) (980 X) f) (2,020 X
clover proliferation (CP) g) (480 X) h) (8,000 X)

X=xylem, (arrow)=phloem, 'MLO'=structures resembling mycoplasmalike organisms, A=amyloplast

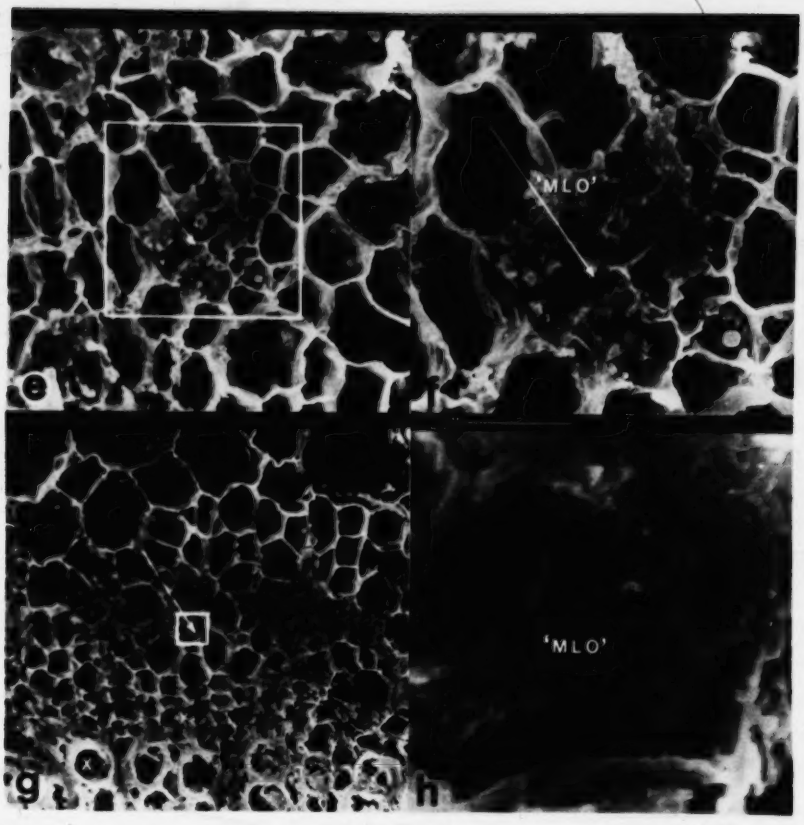

'MLO'
e
'MLO'
g
h

Figure 10 :
Scanning electron micrographs of stem cross-sections of healthy Catharanthus roseus a) (560 X) b) (4,560 X)

X=xylem, (arrow)=phloem

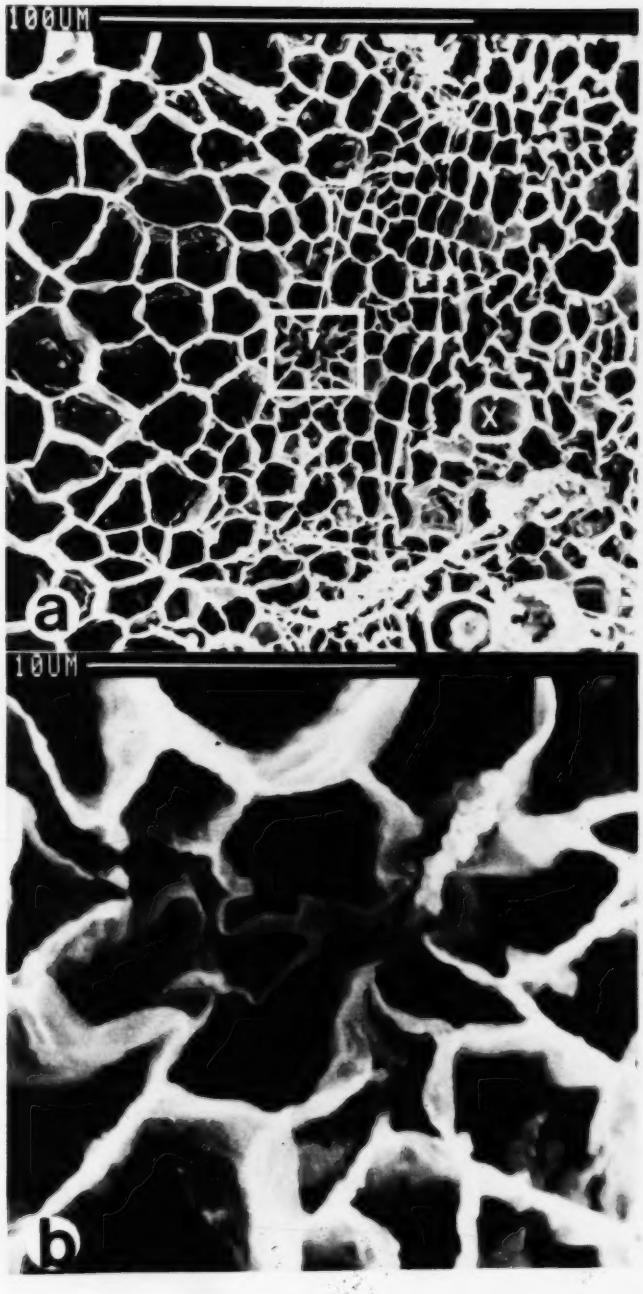

100UM
a
10UM
b

Figure 11 :
Scanning electron micrographs of structures resembling MLOs in the phloem of stem cross-sections of diseased plant sections of Catharnathus roseus infected with MLOs of Eastern aster yellows, New York isolate (EAY), fixed in 3% glutaraldehyde overnight before sectioning
dehydrated in ethanol series starting at 70% a) (240 X) b) (980 X) c) (4,100 X)
dehydrated in ethanol series starting at 35% d) (1,120 X) e) (9,300 X)

X=xylem, (arrow)=phloem, S=sclerenchyma, 'MLO'=structures resembling mycoplasmalike organisms

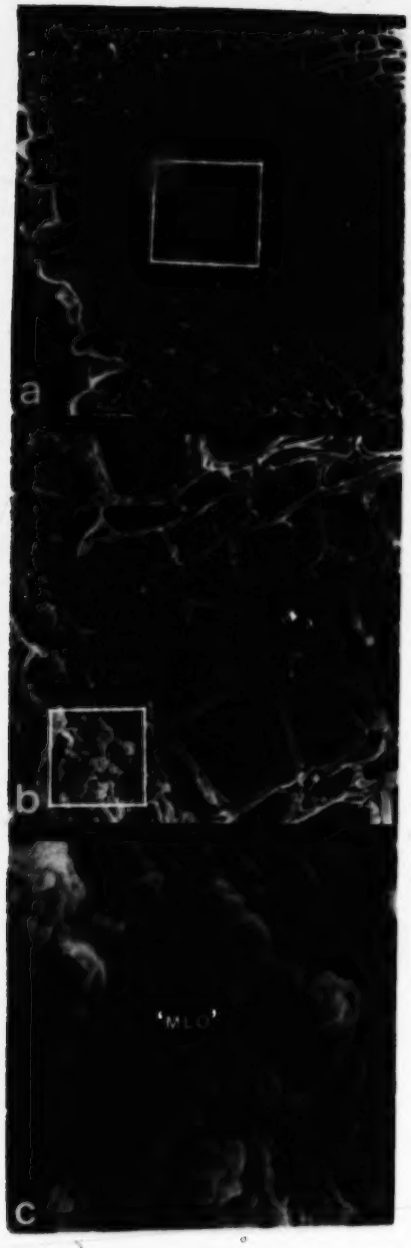

a
b
c

Figure 11 :
Scanning electron micrographs of structures resembling MLOs in the phloem of stem cross-sections of diseased plant sections of Catharnathus roseus infected with MLOs of Eastern aster yellows, New York isolate (EAY), fixed in 3% glutaraldehyde overnight before sectioning
dehydrated in ethanol series starting at 70% a) (240 X) b) (980 X) c) (4,100 X)
dehydrated in ethanol series starting at 35% d) (1,120 X) e) (9,300 X)

X=xylem, (arrow)=phloem, S=sclerenchyma, 'MLO'=structures resembling mycoplasmalike organisms

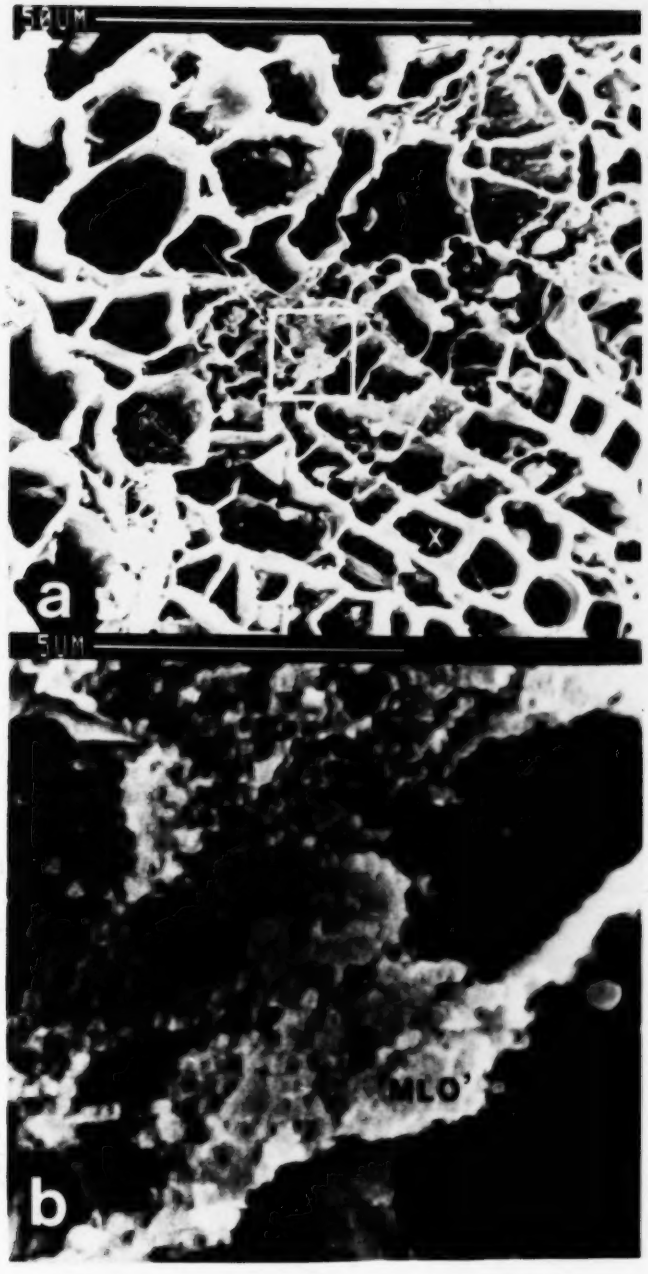
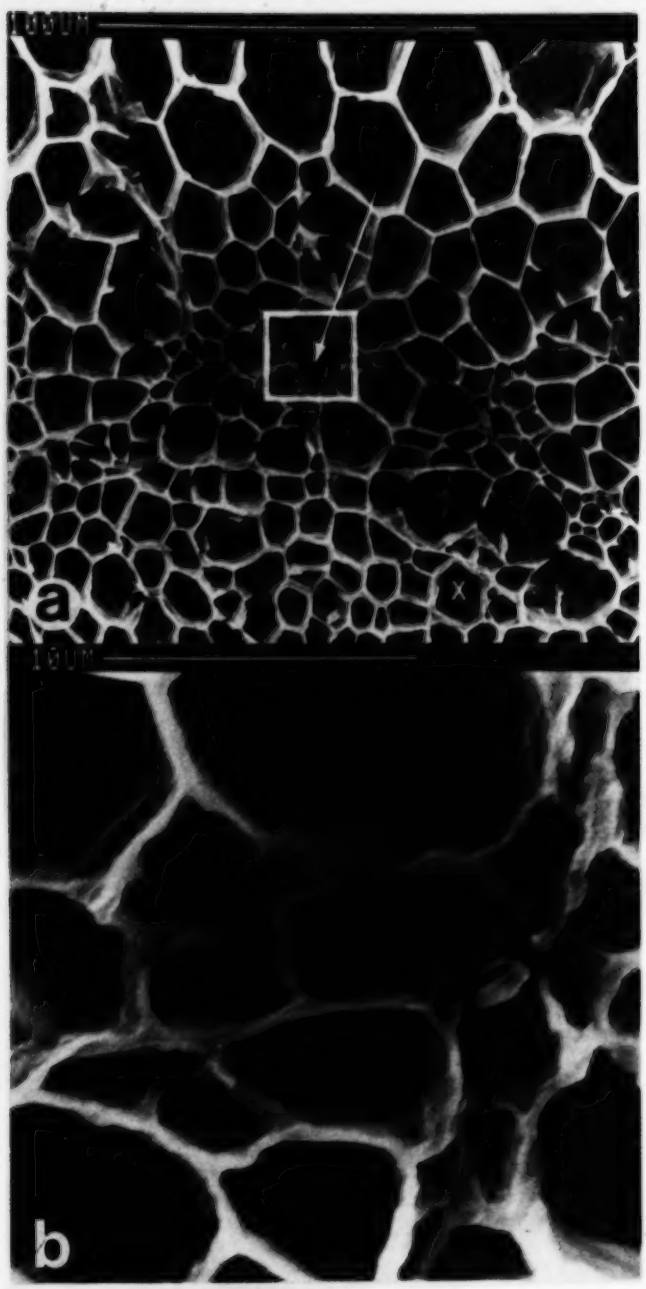

1 µm
a
5 µm
MLO'
b

Figure 12 :
Scanning electron micrographs of the phloem of stem cross-sections of diseased plant sections of Catharanthus roseus infected with MLOs of Eastern aster yellows, New York isolate (EAY), embedded in paraffin before sectioning a) (560 X) b) (4,650 X). No MLOs were observed.

X=xylem, (arrow)=phloem


a
b

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